REMARKS

Claims 38-76 are pending in the application. Applicants request entry and consideration of the amendments and response herein. Accordingly, claims 38 and 40-84 will be pending in the application upon entry of this amendment.

Applicants wish to thank the Examiner for the courtesy extended to their undersigned representative in the interview on December 14, 2004. Further to that discussion, Applicants submit these amendments and response.

Amendment of any claim herein is not to be construed as acquiescence to any of the rejections/objections set forth in the instant Office Action, and was done solely to expedite prosecution of the application. Applicants make these amendments without prejudice to pursuing the original subject matter of this application in a later filed application claiming benefit of the instant application, including without prejudice to any determination of equivalents of the claimed subject mattered. Support for these amendments appears throughout the specification and claims as filed. No new matter is introduced by these amendments.

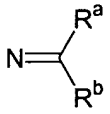
Claim Amendments

Applicants have amended claims 38, 40 and 52. These amendments are made to more clearly delineate the claimed subject matter and correct text errors. No new matter is introduced by this amendment.

Claims 77-82 have been added and are directed towards the treatment of various diseases defined in claim 39. Claims 83 and 84 have been added and are directed towards methods and pharmaceutical compositions comprising a specific compound. No new matter is introduced by this amendment.

Claims Rejections – 35 U.S.C. §112, Second Paragraph

It is alleged that, in claim 38, it is not clear where R^b-N=R^a is attached to, rendering claim 38 indefinite. Applicants have amended the typographical error in claim 38 to show that the R_1 definition includes the following structure, thereby obviating the rejection.



Claims Rejections – 35 U.S.C. §112, First Paragraph

It is alleged that claims 38-39 do not reasonably provide enablement for all disorders generically embraced in claim 38 and other disorders embraced in claim 39. It is further alleged that the scope of the claims includes not only any or all conditions but also those conditions yet to be discovered as mediated by interleukin-12 overproduction, for which it is alleged there is no enabling disclosure. In addition, it is alleged that the Applicants have not provided any competent evidence that the instantly disclosed tests are highly predictive for all the uses disclosed and embraced by the claim language. Applicants disagree and respectfully traverse.

IL-12 overproduction is known to cause excessive Th1 response, and may result in inflammatory disorders, such as rheumatoid arthritis, Crohn's disease, multiple sclerosis, sepsis, and psoriasis. It is therefore indicated that compounds that down-regulate IL-12 production can be used for treating inflammatory diseases.

The Examiner concludes that the specification enables the invention for the treatment of rheumatoid arthritis. Administration of pyrimidine compound 12 reproducibly reduced the arthritic score and delayed development of polyarthritis in a dose-dependent manner in the Adjuvant arthritis model (see Example 29 in the Specification at page 28, line 30). The Examiner has therefore acknowledged that a method of treatment of rheumatoid arthritis is enabled by the instant invention.

The enablement requirement for the treatment of Crohn's disease in claim 39 was not acknowledged by the Examiner. A method of treatment of Crohn's disease in rats was determined by treatment with the pyrimidine compounds of the instant invention. In a Crohn's disease rat model, it was found that compound 12 of the instant invention reduced colonic

inflammation, and that the reduced inflammation could be attenuated with different doses (see Example 29 of the Specification at page 31, line 13). In view of these results, Applicants submit that the enablement requirement to treat Crohn's disease has been satisfied. Further, the reduction in colonic inflammation clearly demonstrates the potential of the pyrimidine compounds of the instant invention in the treatment of inflammatory bowl disease.

In addition, it is known to those of ordinary skill in the art that using compounds to lower Th1 cytokine levels to treat Crohn's disease has enjoyed initial success.[1] Th1 cytokines, such as TNF-α and IL-12, are thought to have a primary role in initiating Crohn's disease, as well as in ongoing inflammatory reactions. The use of anti-TNF-α (antibody) therapy, to reduce the levels of TNF-α, has been utilized in patients with Crohn's disease.[1] A significant clinical response has been observed. Further, the use of anti-IL-12 has been used in an NIH study in the treatment of patients with Crohn's disease.[1] It was found that anti-IL-12 induces clinical responses and remissions of Crohn's disease, and that the treatment was associated with decreases in Th1-mediated inflammatory cytokines.

Because TNF-α and IL-12 are both Th1 cytokines, and treating Crohn's disease using antibodies has enjoyed initial success, one of ordinary skill in the art would appreciate that lowering IL-12 production with pyrimidine compounds of the instant invention could provide similar results. Applicants therefore submit that they satisfy the enablement requirement of 35 U.S.C. §112 for treating Crohn's disease.

The treatment of multiple sclerosis (MS) using antibody treatment is also known to those of ordinary skill in the art.[2] It is also known that in patients with MS, IL-12 secretion is markedly increased. One method of treating MS involves antibody treatment with anti-IL-12, presumably to lower IL-12 levels. In animal models, it has been demonstrated that treating Experimental Autoimmune Encephalomyelitis, EAE (animal model of MS), with anti-IL-12 significantly reduced the EAE severity and incidence and severity of EAE relapse. Further, administration of IL-12 to the animal models with EAE increased the severity of EAE. It is

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[1] (a) Cominelli, F. New England Journal of Medicine (2004), 351, p. 2045. (b) Mannon, P. J. et al. New England Journal of Medicine (2004), 351, p. 2069.

[2] (a) Balashov, K. E. et al. Proc. Natl. Acad. Sci. USA. (1997), 94, p. 599.(b) Constantinescu, C. S. Journal of Immunology. (1998), p. 5097.

therefore indicated that methods which lower IL-12 levels, by antibody treatment or treatment with other compounds/compositions, are useful for treating MS. Based on the foregoing and the fact that Applicants have demonstrated that compounds in the instant application lower IL-12 levels, Applicants submit that the enablement requirement of 35 U.S.C. §112 for treating MS is satisfied.

The potential treatment of diabetes mellitus using antibody treatment is also known to those of ordinary skill in the art.[2,3] It is known that administration of IL-12 induces the rapid onset of insulin-dependent diabetes mellitus in the NOD (non-obese diabetic) mouse. One method of treating diabetes mellitus would involve antibody treatment with anti-IL-12, presumably to lower IL-12 levels. Administration of anti-IL-12 is known to suppress islet destruction, which produces insulin.[4] By suppressing islet destruction, anti-IL-12 is indicated to treat diseases which result from islet destruction, including diabetes mellitus. Based on the foregoing and the fact that Applicants have demonstrated that compounds in the instant application lower IL-12 levels, Applicants submit that the enablement requirement of 35 U.S.C. §112 for treating diabetes mellitus is satisfied.

The treatment of psoriasis using antibody treatment is also known to those of ordinary skill in the art.[5] It is also known that significant amounts of IL-12 are observed in rats with psoriasis. One method of psoriasis treatment involved antibody treatment with anti-IL-12, presumably to lower IL-12 levels. In a psoriatic model that closely resembles human pathology, mice were first subjected to IL-12, and then treated with anti-IL-12. In these mice, psoriasis (or the lesions associated with psoriasis) did not develop, but in the control group (mice treated with an agent that induces psoriasis-like lesions), over 90% of the mice developed psoriasis.[4] It is therefore indicated that methods which lower IL-12 levels, by antibody treatment or treatment with other compounds/compositions, are useful for treating psoriasis. Based on the foregoing and the fact that Applicants have demonstrated that compounds in the instant application lower IL-12 levels, Applicants submit that the enablement requirement of 35 U.S.C. §112 for treating psoriasis is satisfied.

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[3] Trembleau, S. et al. J. Exp. Med. (1995), 181, p. 817.
[4] Ma, L. et al. Diabetes. (2003), 52, p. 1976.
[5] Hong, K. et al. Journal of Immunology. (1999), p. 7480.

The treatment of septic shock (resulting from sepsis) using antibody treatment is also known to those of ordinary skill in the art.[6] It is also known that septic shock results from the release of IFN-γ which is induced by IL-12, which can be induced by LPS (lipopolysaccharide). One method of septic shock treatment involved antibody treatment with anti-IL-12, presumably to lower IL-12 levels. In *in vivo* mouse studies, mice were first subjected to LPS, and then treated with anti-IL-12. In these mice, septic shock, measured by observing IFN-γ levels, was five to sixfold lower than controls (LPS treated mice).[5] It is therefore indicated that methods which lower IL-12 levels, by antibody treatment or treatment with other compounds/compositions, are useful for treating septic shock. Based on the foregoing and the fact that Applicants have demonstrated that compounds in the instant application lower IL-12 levels, Applicants submit that the enablement requirement of 35 U.S.C. §112 for treating sepsis is satisfied.

Thus, methods of lowering IL-12 levels have been indicated to be viable in treating a number of IL-12 overproduction related disorders, such as rheumatoid arthritis, Crohn's disease, MS, diabetes mellitus, psoriasis, and sepsis. Applicants therefore submit that the use of their instant pyrimidine compounds, in view of the knowledge of the results observed using the above-mentioned treatments to lower TNF-α and IL-12 levels, satisfy the enablement requirement of 35 U.S.C. §112 for treating IL-12 overproduction related disorders. Applicants respectfully request withdrawal of this rejection.

Provisional Rejection-Obviousness Type Double Patenting

It is alleged that claims 38-76 of this application (10/656,671) conflict with claims 25-52 of Application No. 10/655,672. Applicants have addressed all other rejections and therefore, pursuant to MPEP 1490, as the provisional obviousness type double patenting rejection is the only rejection remaining and application 10/655,672 is still a pending application, Applicants request withdrawal of this rejection and allowance of this application.

[6] Mattner, F. et al. Infection and Immunity. (1997), p. 4737.

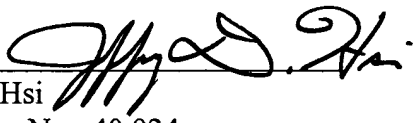
CONCLUSION

In view of the above, reconsideration and withdrawal of all rejections and allowance of the application with claims 38 and 40-84 are respectfully solicited. Accordingly, the Examiner is respectfully requested to pass this application to issue. Should any of the claims not be found to be allowable, the Examiner is requested to telephone Applicants' undersigned representative at the number below. Applicants thank the Examiner in advance for this courtesy.

The Director is hereby authorized to charge or credit any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 04-1105, under Order No. 50586-61248CON.

Respectfully submitted,

Date: December 20, 2004

By 
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Antibodies Against IL-12 Prevent Superantigen-Induced and Spontaneous Relapses of Experimental Autoimmune Encephalomyelitis[1]

Cris S. Constantinescu,[2]* Maria Wysocka,† Brendan Hilliard,* Elvira S. Ventura,* Ehud Lavi,‡ Giorgio Trinchieri,† and Abdolmohamad Rostami[3]*

Immunization of (PL/J × SJL/J)F_1 mice with myelin basic protein (MBP) induces relapsing experimental autoimmune encephalomyelitis (EAE). Relapses occur 7 to 10 days after recovery from the initial paralysis. Staphylococcal enterotoxins (SE) A or B, administered after recovery from the initial paralysis, induce immediate relapses. IL-12 is involved in the induction of EAE. Here, we show that SEA and SEB induce IL-12 in splenocytes from (PL/J × SJL/J)F_1 mice in vitro and increase the level of IL-12 in the sera of mice treated with these superantigens. IL-12 administration mimics SE in inducing spontaneous relapses and in enhancing the severity and frequency of spontaneous relapses. IL-12 neutralization blocks SE-induced and subsequent relapses of EAE, and, when instituted after recovery from the initial attack, prevents spontaneous relapse. This is the first report of prevention of relapses of EAE with anti-IL-12 Ab, an approach which may prove useful in the prevention of exacerbations in multiple sclerosis. *The Journal of Immunology,* **1998, 161: 5097–5104.**

Experimental autoimmune encephalomyelitis (EAE)[4], an animal model for the human disease multiple sclerosis (MS), is a T cell-mediated central nervous system (CNS) autoimmune disease. The autoreactive T cells, directed against neuroantigens including myelin basic protein (MBP), are of the Th1 type (producing IFN-γ and TNF and promoting cell-mediated immunity). These cells preferentially use the Vβ8 TCR in Lewis rats and $H2^u$ mice (1). The superantigen (SAG) staphyloccocal enterotoxin (SE) B (SEB) is a potent T cell activator stimulating a large proportion of Vβ8 T cells and has been postulated to trigger autoimmunity by stimulating autoreactive T cells (2). SEB induces relapsing paralysis in PL/J (3, 4) and (PL/J × SJL/J)F_1 mice (3) that have recovered from the first acute EAE attack. This property of SEB has been attributed to its ability to activate Vβ8 T cells and has given rise to analogies with the frequent precipitation of MS relapses by infectious events (5).

(PL/J × SJL/J)F_1 mice, when immunized with whole MBP, also have a high incidence of spontaneous relapses (6). These typically occur between days 23 and 32 after immunization, (7–10 days after recovery from the initial EAE episode) (6) (C.S.C. and A.R., unpublished observations). Thus, spontaneous relapses in (PL/J × SJL/J)F_1 mice are distinguishable from the SEB-induced relapses (3), which occur within 1 to 3 days after SEB administration. Interestingly, staphyloccocal enterotoxin A (SEA), a SAG that does not activate Vβ8 T cells, can induce similar relapses (3, 4), suggesting that the mechanisms of SAG-induced relapses are not strictly Vβ8-dependent.

Staphylococcal SAG bind the MHC class II molecule of the APC outside of the binding groove and, subsequently, as a binary complex bind to the Vβ region of the TCR (2). This process induces cytokines both in the T cell and in the APC (7). SAG binding to the MHC class II molecule on the APC induces signaling (8), cytokine gene transcription (9) and secretion (10), and nitrite production (11). Staphylococcal SAG preferentially induce Th1 cytokines (12–14). SAG binding is enhanced by IFN-γ, suggesting that cooperation of T cells and MHC class II-positive APC is needed for optimal SAG-induced responses (15).

An important cytokine produced by activated APC is IL-12. This heterodimeric cytokine, consisting of the p35 and p40 subunits, induces IFN-γ production by T and NK cells (16, 17) and is pivotal in Th1-type immune response development (18, 19). IL-12 is involved in the induction of the acute phase of EAE, as demonstrated by the ability of a neutralizing anti-IL-12 Ab to prevent both actively induced EAE (C.S.C. and A.R., unpublished observations) and adoptively transferred EAE (20), and by the increased encephalitogenicity of neuroantigen-reactive T cells stimulated with IL-12 (20, 21). In addition, exogenous IL-12 induces relapses in an otherwise typically monophasic form of EAE in Lewis rats (22). Thus, it is of interest to determine whether endogenous IL-12 plays a role in EAE relapse, characterized in our mouse model by both spontaneous and SAG-induced relapses. Moreover, because SAG, including staphylococcal SAG, can induce IL-12 production by APC in vitro (23–25), it is interesting to determine this ability in vivo (particularly because SAG may have different effects in vitro and in vivo) and to investigate whether this induction plays a role in the relapsing paralysis of EAE.

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[4] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; CNS, central nervous system; MBP, myelin basic protein; SAG, superantigen; SE, staphyloccocal enterotoxin; SEB, staphyloccocal enterotoxin B; SEA, staphyloccocal enterotoxin A.

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Staphylococcus aureus is a potent inducer of IL-12 (26). Moreover, recent evidence indicates that the ability of several bacteria-derived substances, including staphylococcal products, to overcome resistance to EAE is mediated through IL-12 induction (27). *S. aureus* SAG also synergize with IL-12 (28). In this study, we investigated the role of IL-12 in relapsing EAE in (PL/J × SJL/J)F_1 mice. We analyzed the p40 subunit (the inducible component of IL-12) and the p70 heterodimer (generally p40 correlates well with the biologically active p70) (16, 26). We showed that SE induce IL-12 in splenocytes in vitro and in vivo. We also demonstrated that neutralizing anti-IL-12 Abs prevent SE-induced relapses. Further, spontaneous relapses are prevented by anti-IL-12 Abs. IL-12 administration mimics SE in inducing spontaneous relapses and enhancing the severity and frequency of spontaneous relapses.

Materials and Methods

Mice

Male or female (PL/J × SJL/J)F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were sex and age matched within each experiment. Previous studies in our laboratory and others' (6) showed no differences between male and female mice of this strain in terms of EAE susceptibility and frequency of relapses.

Reagents and Ag

MBP was prepared from guinea pig spinal cord (Rockland, Gilbertsville, PA) (29), lyophilized, and stored at −20°C until use. SEA and SEB were purchased from Sigma (St. Louis, MO). Recombinant murine IL-12 was a generous gift of Dr. Maurice Gately (Hoffmann-La Roche, Nutley, NJ). Murine rIFN-γ was purchased from PharMingen (San Diego, CA). Monoclonal anti-mouse IL-12 Abs C17.8 (rat IgG2a), C15.1 (rat IgG1), and C15.6 (rat IgG1) were previously described (30). IFA and *Mycobacterium tuberculosis* H37 Ra were purchased from Difco (Detroit, MI). A 1:1 mixture resulted in CFA with 5 mg/ml *M. tuberculosis*. *Bordetella pertussis* toxin was obtained from List Biological Laboratories, (Campbell, CA). Rat Ig was purchased from Sigma.

Induction of EAE

Mice, 10 wk old, under anesthesia were immunized on day 0 in the hind footpads and at three sites on each side of the back with a total of 300 μl of 1:1 (v/v) mixture of MBP (2.67 mg/ml in PBS) and CFA containing 5 mg/ml *M. tuberculosis* H37Ra. On days 0 and 2, 400 ng *B. pertussis* toxin were given i.p.

Clinical scoring

Mice were weighed daily and observed for clinical signs of disease for more than 40 days postimmunization. A clinical scoring system with a scale of 0 to 5, with 0.5 points for intermediate signs, was used as follows: 0, normal; 1, flaccid tail, abnormal gait; 2, hind leg weakness or severe ataxia; 3, minimal hind leg movement; 4, hind leg and forelimb paralysis; 5, moribund due to EAE, with impaired breathing and little or no spontaneous movement. Mild disease had to be observed for 2 days or more and be confirmed by two independent, blinded observers to be considered positive. The score increment was defined as the maximal increase, during a relapse, in the clinical score from the prerelapse score.

Induction of relapses and treatment after recovery from first EAE episode

Mice that had recovered from the acute disease (day 18 or 19 postimmunization) were injected i.p. with a single dose of 50 μg SEB or 25 μg SEA, both in PBS, or with 100 ng murine rIL-12 in PBS with 1% mouse serum. On the day of the above treatment and on the subsequent 2 days, some of the mice received 1 mg/day of either anti-IL-12 mAb C.17.8 or control rat IgG.

The mice were observed daily for development of relapsing disease. Relapses occurred 1 to 3 days after SE administration. Relapses with onset occurring 3 to 5 days following the treatment were attributed to the intrinsically relapsing nature of EAE in these mice and coincided with relapses in mice that had been immunized on the same day but received no treatment following recovery from the initial episode. However, we cannot rule out the possibility that relapses occurring during this period may also be related to the effect of the SAG in the SAG-treated animals.

Measurement of cytokines in serum and splenocyte supernatants

Mice were given the above doses of SEA or SEB in 100 μl PBS via i.p. injection. Control mice were given 100 μl PBS. Blood was harvested in heparinized tubes by retro-orbital bleeding at 6 h and 24 h, centrifuged, sera removed and diluted at concentrations of 1:10 for IL-12 p40 and IL-12 p70 measurement.

For in vitro cytokine studies, spleens of mice were homogenized to single-cell suspensions by passage through a stainless steel mesh. RBC were removed by hypotonic lysis in NH_4Cl-containing buffer. IL-12 p40 and IL-12 p70 assays were performed in total spleen cell populations stimulated either with medium alone (RPMI 1640 with 5% FBS with antibiotics) or with the same medium containing SEA or SEB, 2.5 μg/ml. IL-12 p40 was assayed in supernatants of spleen cells of mice using recombinant murine IL-12 as standard, following a two-site RIA as described (30). Briefly, samples were placed in 96-well plates (Dynatech Laboratories, Chantilly, VA) coated with 5 μg/ml of C17.15 anti-mouse IL-12 mAb and incubated overnight at 4°C. Plates were washed, and ^{125}I-labeled C15.6 was added. Bound radioactivity after 6 h incubation at 4°C was measured in a microplate scintillation counter (Topcount, Packard, Meriden, CT). Samples were assayed in triplicate. IL-12 p70 was measured in a biologic assay based on IL-12-dependent induction of IFN-γ in murine splenocytes, as described (30). The sensitivity of RIA detecting IL-12 p40 is 30 pg/ml, and the sensitivity of biologic assay detecting IL-12 p70 is 3 pg/ml. IFN-γ was assayed in supernatants of spleen cells of mice using recombinant murine IFN-γ as standard according to a two-site RIA as described (30).

Histopathology

Animals under anesthesia were perfused through the heart with 10% phosphate-buffered formalin. Brains and spinal cords were fixed in formalin, dehydrated through graded alcohols, and embedded in paraffin. Five-micrometer sections of brain and spinal cord were cut serially and mounted on poly-L-lysine-coated glass slides. Five spinal cord sections and five brain sections obtained at similar levels from each mouse were stained. For assessment of inflammation, sections were stained with hematoxylin-eosin. For evaluation of demyelination, sections were stained with Luxol fast blue and counterstained with cresyl violet. The extent of inflammation and demyelination was scored on a scale of 0 to 3, based on the fraction of tissue section quadrants containing lesions out of 20, as previously described (31): 0, absent; 1, mild (1–7 quadrants); 2, moderate (8–13 quadrants); 3, severe (14–20 quadrants); and 0.5 points for intermediate degrees of histologic severity.

Statistical analysis

The two-tailed Student's t test was used to assess the significance of differences in clinical scores between groups. Incidences of EAE relapses were compared using the χ^2 test.

Results

SE induce IL-12 in vitro

To determine whether SE induce IL-12 production by splenocytes (most likely APCs) in vitro, we harvested spleen cells from naive (PL/J × SJL/J)F_1 mice and measured the production of IL-12 p40 in cell culture supernatants 6 and 24 h after stimulation with SE in vitro, with or without IFN-γ (100 U/ml) pretreatment. Both SEA and SEB induced IL-12 p40 in (PL/J × SJL/J)F_1 splenocytes without IFN-γ pretreatment (data not shown), confirming results of studies using murine peritoneal macrophages (24). This induction was consistently higher when splenocytes were pretreated with IFN-γ (100 U/ml) for 16 to 18 h before stimulation. Figure 1*A* shows the results of a typical experiment, in which induction of IL-12 p40 by SEA and SEB is demonstrated in murine splenocytes after pretreatment with IFN-γ. Without IFN-γ pretreatment, the corresponding IL-12 p70 measured by biologic assay was either just above the detection limit (≤2 pg/ml) or absent. In contrast, IFN-γ pretreatment resulted in consistently detectable amounts of IL-12 p70 after SE stimulation. Figure 1*B* shows an example of IL-12 p70 induction by SEA and SEB after IFN-γ pretreatment measured in the same supernatants as in Figure 1*A*. Stimulation with IFN-γ alone did not induce significant levels of IL-12 p40 or IL-12 p70 (data not shown). The slight increase in IL-12 p40 after 24 h in culture (as seen in Fig. 1*A*) is also noted with splenocytes

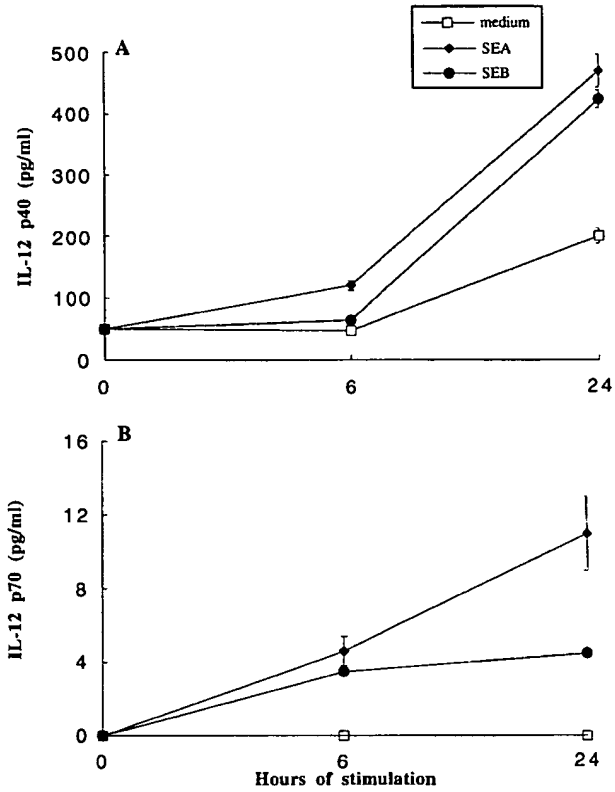

FIGURE 1. Production of IL-12 p40 (*A*) and IL-12 p70 (*B*) by murine spleen cells prestimulated in vitro with IFN-γ (100 U/ml) for 16 h and then stimulated with 2.5 μg/ml SEA or SEB for the designated durations. Results are shown as mean ± SEM of triplicate measurements of one experiment. Splenocytes were obtained and pooled from two to three (PL/J × SJL/J)F_1 mice per experiment. Similar results were obtained in four additional experiments.

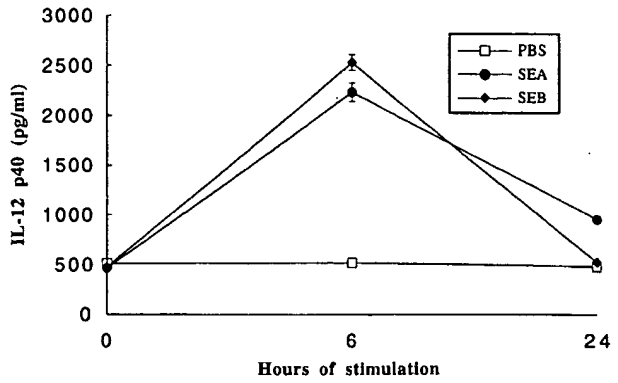

FIGURE 2. Induction of IL-12 p40 by SE in vivo. Mice were injected with SEA 25 μg or SEB 50 μg i.p. Heparinized blood was harvested at the designated time points, and IL-12 p40 levels were measured in diluted serum samples by RIA as described in *Materials and Methods*. Results are shown as mean ± SEM of three measurements per time point, each obtained from the serum of one mouse. Similar results were obtained in three additional experiments.

that were allowed to adhere for ≤24 h without IFN-γ or other stimuli; this increase is never associated with detectable IL-12 p70.

To determine whether IL-12 induction by SE correlates with induction of IFN-γ, we measured IFN-γ in supernatants of SE-stimulated spleen cells. Large amounts of IFN-γ, up to 40 ng/ml, were detected after both SEA and SEB stimulation, the peak of IFN-γ corresponding to that of IL-12 p40 and IL-12 p70 at 24 h after stimulation (data not shown).

SE induce IL-12 in vivo

The in vitro and in vivo effects of SAG can be different, particularly with respect to T cell activation vs anergy. In contrast, SE are known to induce cytokines, including TNF, both in vivo and in vitro (32). However, although neutralization of TNF alone significantly delayed SEB-induced relapses, it did not completely prevent them (3). IL-12 is involved in EAE induction (20) and synergizes with TNF (33, 34). Therefore, we investigated whether in vivo SE administration increases IL-12. We measured serum IL-12 p40 in mice 6 and 24 h after the i.p. administration of 25 μg/mouse SEA or 50 μg/mouse SEB. We demonstrated that this treatment induces increased levels of p40 IL-12 in the sera of these mice (Fig. 2). The induction kinetics is similar to that shown in murine endotoxic shock (30). We also measured the levels of IL-12 p70 heterodimer in these sera and observed consistently detectable levels only at the 6-h time point (data not shown).

SE-induced relapses are prevented by anti-IL-12 Ab

Previous studies have shown that anti-IL-12 Abs prevent acute monophasic EAE, while IL-12 increases its severity (20). We examined whether the neutralizing anti-IL-12 mAb C17.8 could inhibit or prevent SEB- or SEA-induced EAE relapses. After recovering from the initial acute EAE episode (day 18 or 19), mice received SEA 25 μg/mouse or SEB 50 μg/mouse i.p. On the day of SE treatment and on the following 2 days, a group of mice received additional treatment with anti-IL-12 mAb (1 mg/mouse i.p. per treatment), while the control group received control rat IgG (same dose). As shown in Table I and Figure 3, the incidence and severity (measured as mean increment in clinical score and mean maximal score) of SAG-induced EAE relapses was significantly reduced ($p < 0.001$ for both SEA and SEB) by treatment with anti-IL-12 Ab, while treatment with control rat IgG was ineffective.

SE enhance severity and frequency of EAE relapses

The above results confirm the ability of SE to induce clinical relapses in (PL/J × SJL/J)F_1 mice. In addition, this strain of mice

Table I. *Effect of anti-IL-12 on SE-induced EAE relapse*[a]

	Treatment					
	SEB	SEB + αIL-12	SEB + rat IgG	SEA	SEA + αIL-12	SEA + rat IgG
Incidence of relapses (%)	11/12 (91.6)	0/12 (0)[b]	7/7 (100)	6/8 (75)	0/8 (0)[b]	3/5 (60)
Mean score increment (SD)	1.27 (0.44)	0 (0)[b]	1.21 (0.26)	0.83 (0.12)	0.06 (0.1)[b]	0.7 (0.1)
Mean maximal score (SD)	1.54 (0.43)	0.23 (0.14)[b]	1.31 (0.26)	0.92 (0.71)	0.12 (0.18)[b]	0.7 (0.44)

[a] Mice were immunized with MBP + CFA. After recovering from the initial acute EAE episode (day 18 or 19), mice received SEB (25 μg) or SEA (50 μg). On the day of SE treatment and on the following 2 days, one group of mice received anti-IL-12, while a control group received rat IgG.

[b] Significant difference with control rat IgG treatment ($p < 0.001$).

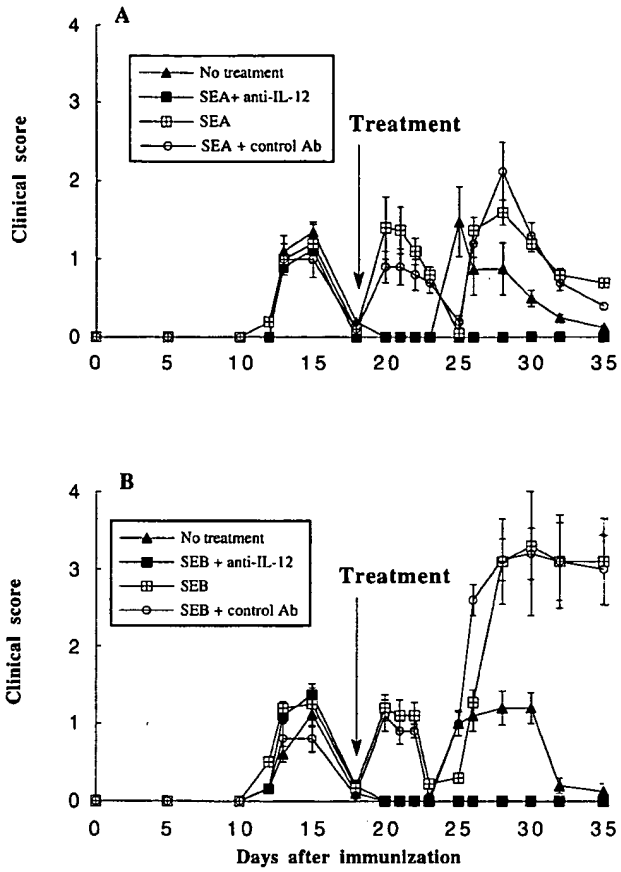

FIGURE 3. Prevention of SEA-induced (*A*), SEB-induced (*B*), or spontaneous EAE relapses (shown both in *A* and *B* in the No-treatment groups) by neutralizing anti-IL-12 mAb. After recovery from the initial EAE attack, mice were either given no further treatment or were treated as indicated in the figure by the arrow and the notation "Treatment." Results are shown as mean ± SD of clinical scores given as described in *Materials and Methods*. The differences between SE only or SE + control Ab- and SE + anti-IL-12 Ab-treated mice are statistically significant ($p < 0.05$). Further characteristics of EAE in each group are given in Tables I and II. The number of mice shown in the figure for each experimental group corresponds to the total number per group shown in Tables I and II.

has a tendency toward spontaneous relapses, with incidence varying between 20% (3) and 100% (6), depending in part on the immunization protocol. Our unpublished results have shown a mean incidence of spontaneous relapses of 75% (range: 50–100%), with typical occurrence 7 to 10 days after recovery from the initial paralysis. In this paper, we use the term "spontaneous relapses" to denote relapses that occur in immunized mice that have received neither SE nor IL-12 treatment after the recovery from their initial EAE episode. We examined whether SE administration influences the occurrence or severity of spontaneous relapses. We found that animals treated with SE after recovery from the initial EAE event, in addition to experiencing an immediate SE-induced relapse, had an increased incidence and severity of later relapses ($p < 0.0001$) (Table II and Fig. 3). Anti-IL-12 Ab treatment at the time of SE administration significantly decreased the incidence and severity of relapses, while administration of the control Ab rat IgG at the time of SE administration had no effect.

Histopathologic evaluation of the brain and spinal cord was performed on selected mice from the SE + control Ab- and SE + anti-IL-12-treated groups. Mice were sacrificed at day 33 postimmunization. SE + control Ab-treated mice exhibited mild perivascular and meningeal inflammation in the brain and severe inflammation involving the meninges, the perivascular spaces, and parenchyma, and accompanied by demyelinating changes in the spinal cords (Fig. 4, *A* and *B*). In contrast, histopathology in SE + anti-IL-12-treated mice revealed no inflammatory or demyelinating changes (Fig. 4*C*), with the exception of minimal meningeal infiltration in a single case out of three in SEB + anti-IL-12-treated mice (Table III). The differences between SE + control Ab and SE + anti-IL-12 were statistically significant ($p < 0.05$).

Severity and frequency of EAE relapses are enhanced by IL-12 and suppressed by anti-IL-12 Ab

We postulated that anti-IL-12 treatment may suppress relapses in mice not given SE treatment. We also wanted to determine whether systemic administration of murine rIL-12 can mimic SE effects by inducing EAE relapses and/or affecting the severity of relapses. After recovery from the initial bout of EAE, five mice received anti-IL-12 mAb C17.8 (1 mg/mouse/day i.p. for three consecutive days) while four control mice received the same quantities of rat IgG. None of the anti-IL-12-treated mice developed relapses, whereas three of four (75%) control Ab-treated mice had relapses. The mean severity of the relapse (±SD) in the rat IgG-treated group was 1.125 (±1.031); the mean increment was 1.125 (±0.25). The difference was statistically significant ($p = 0.04$ compared with the rat IgG-treated group) (Fig. 5). The protective effect of anti-IL-Abs was longer lasting than the ~15 days of persistence of these Abs in the circulation. None of the five animals followed ≥50 days developed a relapse, in contrast to the average of 60% of untreated mice that developed at least a third attack.

After recovery from the initial EAE episode (day 18 or 19), 10 mice received 100 ng i.p. of murine rIL-12. Six mice (60%) developed a relapse on the day following IL-12 administration (mean ± SD score increment, 1.08 ± 0.44). No relapses were noted immediately following the cytokine treatment in the remaining four mice. However, all mice receiving IL-12 treatment had a significantly more severe relapse. These relapses occurred at the same time (day 24–30) as the spontaneous relapses in mice not receiving treatment after recovery from the initial paralysis. However, the incidence (10/10 or 100%) was higher than that of spontaneous relapses in the animals without this treatment (6/9 or

Table II. *Effect of anti-IL-12 on SE-enhanced spontaneous EAE relapses*[a]

	Treatment						
	None	SEB	SEB + αIL-12	SEB + rat IgG	SEA	SEA + αIL-12	SEA + rat IgG
Incidence of relapses (%)	6/9 (66.7)	12/12 (100)	2/12 (16.7)[b]	7/7 (100)	7/8 (87.5)	0/8 (0)[b]	4/5 (80)
Mean score increment (SD)	1.08 (0.2)	3.57 (1.2)	0.18 (0.38)[b]	2.5 (1.4)	2.31 (1.7)	0.08 (0.2)[b]	2.6 (1.65)
Mean maximal score (SD)	1.11 (1.54)	3.64 (1.4)	0.2 (0.4)[b]	2.93 (1.6)	2.43 (1.7)	0.25 (0.24)[b]	2.6 (0.24)

[a] Mice were immunized with MBP + CFA. After recovering from the initial acute EAE episode (day 18 or 19), mice received SEB (25 μg) or SEA (50 μg). On the day of SE treatment and on the following 2 days, one group of mice received anti-IL-12, while a control group received rat IgG.

[b] Significant difference with control rat IgG treatment ($p < 0.0001$).

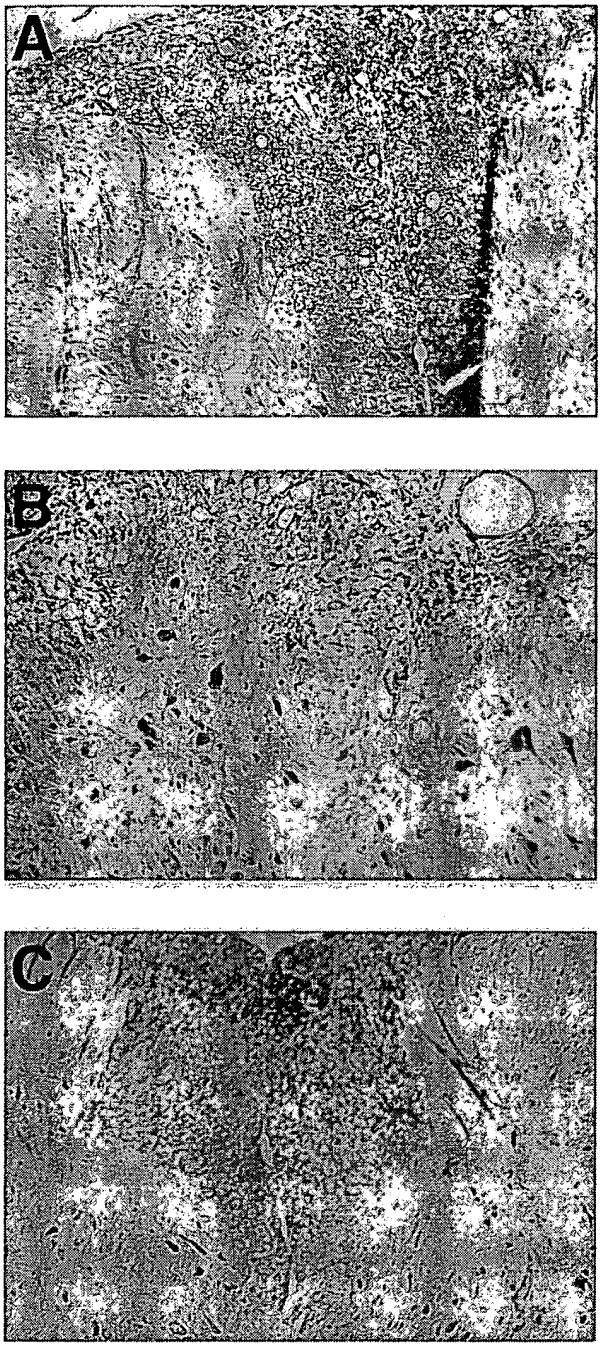

FIGURE 4. Representative section of spinal cord of mouse with EAE after administration of SEB + control Ab (*A* and *B*) or SEB + anti-IL-12 Ab (*C*). Perivascular and parenchymal inflammation and demyelination are noted in *A* and *B*, while no significant histopathology is observed in *C*. Similar results were obtained in mice with SEA-induced relapses. The differences between SE + control Ab- and SE + anti-IL-12 Ab-treated mice were statistically significant ($p < 0.05$). This section was stained with Luxol fast blue and counterstained with cresyl violet. Magnification: *A* and *C*, ×200; *B*, ×400.

67%). In addition, the mean (±SD) score increment was 2.61 (±1.53), as compared with 1.08 (±0.2) in the untreated mice. Also, the mean (±SD) maximal scores were enhanced: 2.71 (±1.44) as compared with 1.11 (±1.54) in mice receiving no IL-12 treatment. The difference was significant ($p < 0.05$ compared with mice receiving no treatment).

Table III. *Histopathology of EAE in SE + control Ab and SE + anti-IL-12 Ab-treated mice*

	Treatment			
	SEA + rat IgG	SEA + α IL 12	SEB + rat IgG	SEB + α IL-12
Inflammation				
Brain	0.5 ± 0	0 ± 0*	0.67 ± 0.17	0 ± 0†
Spinal cord	2.75 ± 0.25	0 ± 0*	2.5 ± 0.29	0.17 ± 0.17‡
Demyelination				
Brain	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Spinal cord	0.67 + 0.17	0 + 0§	1.0 + 0.29	0 + 0¶

[a] Three mouse brains or spinal cords were included in each treatment group, with the exception of spinal cords of SEA + anti-IL-12-treated mice, for which $n = 2$. Histopathologic evaluation was performed, and scores were given as described under *Histopathology*.

*, $p \leq 0.001$; †, $p \leq 0.02$; ‡ $p \leq 0.002$; §, $p = 0.05$; ¶, $p \leq 0.03$.

Discussion

These studies indicate that IL-12 plays a role in spontaneous and SE-induced EAE relapses. In our investigation of expression of IL-12 during murine relapsing EAE in (PL/J × SLJ/J)F_1 mice, we have found mRNA for IL-12 in the CNS only during the acute phases and its absence during remission phases (C.S.C. and A.R., unpublished observations). When we measured serum IL-12 during various stages of EAE in these mice, we did not find detectable levels of IL-12 p70. We believe that the amount of IL-12 in the serum of these animals is below the threshold of detection of our assay and will increase to detectable levels when mice are treated with SE or IL-12. The fact that neutralization of endogenous IL-12 prevented relapses argues for an important role of endogenous IL-12 in the course of EAE.

IL-12 is essential in the generation of Th1 responses and, therefore, plays a key role in the immune response to intracellular pathogens (19). Moreover, IL-12 is involved in the induction of T cell-mediated autoimmune diseases (35) including EAE (20, 21, 27). The role of IL-12 in the maintenance or recurrence of Th1

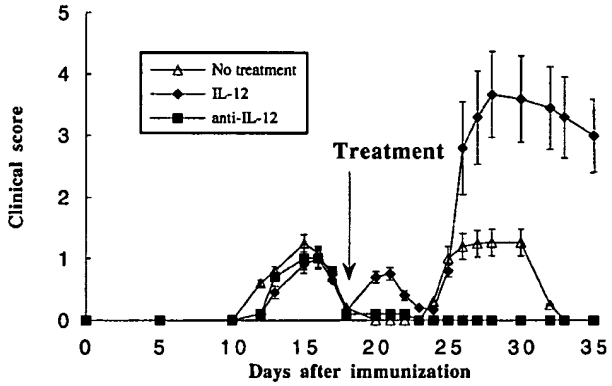

FIGURE 5. Effect of IL-12 and of neutralizing anti-IL-12 mAb on the course of relapsing EAE. After recovery from the initial attack of EAE, mice were treated as indicated in the figure by the arrow and the notation "Treatment." IL-12 mimics in part SE action by inducing immediate relapses and worsening later relapses. Anti-IL-12 Ab prevents spontaneous relapses. Results are shown as mean ± SD of clinical scores given as described in *Materials and Methods*. The course of EAE in mice treated with control rat IgG completely overlaps with that of mice receiving no treatment and is omitted for graphical clarity. The number of mice in each group is as follows: anti-IL-12 mAb, $n = 5$; rat IgG, $n = 4$; IL-12, $n = 10$; no treatment, $n = 10$). Further characteristics and statistical data are included in *Results*.

immune responses has also become a subject of recent investigations. The memory responses to certain infections appear to be IL-12-independent (36, 37). In the well-characterized model of murine leishmaniasis, we also recently demonstrated IL-12 independence of the Th1 response in the secondary infection (69). In contrast, the maintenance and chronicity of some, but not all, T cell-mediated autoimmunity appear to require IL-12. For example, the established chronic autoimmune reaction in experimental colitis is abrogated by neutralization of IL-12 (38). In contrast, however, administration of IL-12 during the established phase of autoimmune collagen-induced arthritis suppressed disease due to induction of IL-10 (39). Seder (40) has hypothesized that the differential IL-12 dependence of established Th1 responses in autoimmunity vs infectious diseases may reflect differences in the initial levels of endogenous IL-12 induction, with higher amounts of IL-12 produced during infection than during the response to an autoantigen. Because IL-12 also induces IL-10 (41, 42) in a potential autoregulatory loop, it is possible that the differential IL-12 dependence resides in the balance between IL-12 and IL-10. The role of IL-10 in EAE has been debated. A role in the recovery from EAE has been postulated based on the presence of IL-10 mRNA in the CNS of EAE animals in the recovery phase (43). With regard to the induction phase of EAE, the exogenous delivery of the cytokine in some studies was successful (44), while in other studies it was unsuccessful in preventing the disease (45). With regard to relapses, exogenous IL-10 could not prevent the SEB-induced relapses, while neutralization of the endogenous IL-10 worsened the relapses (46). This underscores the strength of cross-regulation in the endogenous cytokine network, which can determine the susceptibility, the course, and the cytokine dependence of autoimmunity. Recently, a unique IL-10/IL-12 immunoregulatory circuit controlling susceptibility to EAE has been demonstrated (47), and it is likely that perturbation of this loop (for example, as in our study) by microbial products powerfully changes the outcome and manifestations of EAE.

A clear possibility to consider in the case of relapsing autoimmunity is that IL-12 is important for the phenomenon of determinant (epitope) spreading. Evidence has accumulated indicating that propagation and reactivation of autoimmune diseases occur through the acquisition of autoreactivity to new self-determinants (48–50). Recently it has been shown that during the development of the determinant spreading cascade in murine relapsing EAE new sets of T cells are generated that exhibit a Th1 phenotype (50). Therefore, it is likely that the presence of IL-12 in the cytokine milieu during epitope spreading facilitates the development of these pathogenic neoautoreactive T cells.

Optimal Th1 responses require a synergy between IL-12 and the B7-CD28 interaction (51, 52). Blockade of B7 costimulation effectively prevented epitope spreading and clinical relapses in EAE (53, 54). Therefore, it is also important to characterize the role of IL-12, the other principal component required for optimal Th1 responses, in ongoing chronic or relapsing T cell-mediated autoimmunity. Here, we show that IL-12 neutralization can also prevent spontaneous relapses in EAE. In addition, we effectively prevented the occurrence of SE-induced relapses with an anti-IL-12 Ab. Although the assessment of the longevity of the protective effect of IL-12 neutralization was not the primary objective of this study, the fact that animals given anti-IL-12 Ab did not develop relapses for a long time following treatment while untreated animals did, provides further support to the hypothesis that once epitope spreading is prevented through blockade of costimulation and/or of IL-12, relapses are also prevented.

The mechanisms of SAG-induced and spontaneous relapses may be different. Activation of the residual SAG-responsive T cells with specific Vβ TCR (for example, Vβ8 for SEB) (3) is postulated and likely to be responsible for the SE-induced relapses. Epitope spreading, as discussed above, is involved in spontaneous EAE relapses (45). Our current findings implicate IL-12 in the mechanism of both processes of T cell reactivation and corresponding clinical relapse in EAE. With respect to the SE-induced relapses, we demonstrated that SE induces IL-12 in vitro, consistent with previous results (24), and, to our knowledge, documented for the first time induction of IL-12 by SAG in vivo. Moreover, we showed that exogenous IL-12 mimicked SE action, inducing rapid relapses, resembling effects recently shown in Lewis rats (22). Because staphylococcal SAG also induce IFN-γ (55), it is possible that IFN-γ production by T cells is stimulated by SE-induced IL-12, and reciprocal stimulation of IFN-γ and IL-12 between T cells and APC is initiated. Although both SE induced similar levels of IL-12 in vitro and in vivo, the severity of SEA-induced relapses was lower than that of relapses induced by SEB. However, both SEA- and SEB-induced relapses exhibited significant dependence of this IL-12 induction. The basis of these differences is currently not completely elucidated. We used the same doses of SE that were shown in a previous study (3) to induce relapses in (PL/J $\times$ SJL/J) mice. Interestingly, in that study, SEA was also less efficient than SEB in inducing relapses. It is possible that, despite similar IL-12 inducibility, the lower frequency of SEA-responsive T cells makes this reciprocal stimulation between IL-12 and IFN-γ less efficient, which may explain the lower severity of SEA-induced relapses.

Migration of T cells to the inflammatory compartment, in this case the CNS, may also be stimulated by SE via IL-12 in a manner similar to the demonstrated IL-12 mediation of skin-homing receptor induction by staphylococcal SAG (23). This can explain the absent or minimal inflammation or demyelination in the CNS of mice given SE + anti-IL-12 compared with the inflammatory infiltrates of mice given SE + control Ab observed in our study. IL-12 up-regulates the very late Ag (VLA)-4-dependent T-cell migration (56), a phenomenon known to be required for T cell entry into the brain parenchyma (57). We also demonstrated enhanced expression of VLA-4 on murine MBP-reactive T cells after exposure to IL-12 (C.S.C. and A.R., unpublished observations). Thus, the neutralization of IL-12 may have prevented the up-regulation of VLA-4 and the reentry of autoreactive T cells into the brain. In addition, IL-12 may provide a death-preventing signal to MBP reactive T cells, as shown for other Ag-specific T cells (58).

No or very few residual inflammatory cells were seen in the CNS of mice given SE and treated with anti-Il-12 Abs. Because the time elapsed after recovery from the initial attack was relatively short, one may have expected residual inflammatory infiltrates. Their absence could be interpreted as a stimulated efflux of inflammatory cells from the CNS of mice treated with anti-IL-12 Ab similar to that postulated in the case of EAE mice treated with altered peptide ligands in which relapses were also prevented (59). Another explanation, consistent with our observation that the first bout is relatively short and mild in these mice and with the fact that the clinical residual deficit after the first bout was also mild, is that the inflammation was almost completely resolved at the time of SE administration. In support of this explanation is the fact that histologic analysis of the CNS of mice with near-complete clinical recovery shortly after the first episode is usually normal or only minimally abnormal (C.S.C., A.R., and B.H., unpublished observations).

Because spontaneous relapses may occur through epitope spreading (49), our results suggest that IL-12 plays a role in this phenomenon. Because B7 blockade also prevents spontaneous relapses, this is additional evidence for the complementarity of these

two costimulatory factors necessary for optimal Th1 responses in vivo and in vitro.

Previously, other cytokine-based immunologic manipulations have affected either SAG-induced or spontaneous EAE relapses. Administration of TGF-β (46, 60, 61), IL-10 (46), or IFN-τ (62) prevented relapses, while anti-TGF-β worsened them (63). Blockade of TNF decreased the incidence and severity of relapses (3, 63) and ameliorated EAE in a chronic/relapsing model (64). Interestingly, the cytokines that can prevent relapses antagonize IL-12, while cytokines implicated in relapse pathogenesis synergize with IL-12 (33, 65, 66). Thus, disease-suppressing effects in relapsing EAE may be mediated through IL-12 blockade, while relapses may involve IL-12 and synergistic factors.

Triggering and reactivation of autoimmunity by infectious products are documented in both spontaneous and experimental disease. Spontaneous EAE in MBP-specific transgenic mice occurred only in animals kept in a normal, pathogen-containing environment and not in animals kept in a specific pathogen-free facility (67). The effects of SAG on EAE exacerbations have been attributed either to restricted TCR-dependent mechanisms (3) or to nonspecific mechanisms, which include cytokine release (4). The present study supports the latter hypothesis that SAG also employ nonspecific mechanisms in reactivating EAE. The role of cytokines in the infection-induced reactivation of autoimmunity has been investigated, particularly with respect to TNF (3, 46, 68). Segal et al. (27) recently implicated IL-12 in the pathogenesis of this phenomenon as well. Our results extend these observations and bring them into the context of bacterial T cell SAG.

In conclusion, we show the ability of SE to induce IL-12 and demonstrate the involvement of IL-12 in SE-induced and spontaneous relapses of EAE. These findings may help to elucidate immunopathogenic mechanisms of relapsing autoimmunity and may provide clues to the immunopathology of MS. Neutralization of IL-12 may prove an effective therapy for autoimmune demyelination.

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References

1. Zamvil, S. S., and L. Steinman. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol. 8:579.*
2. Marrack, P., and J. Kappler. 1990. The staphylococcal enterotoxins and their relatives. *Science 248:705.*
3. Brocke, S., A. Gaur, C. Piercy, A. Gautam, K. Gijbels, C. G. Fathman, and L. Steinman. 1993. Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. *Nature 365:642.*
4. Schiffenbauer, J., H. M. Johnson, E. J. Butfiloski, L. Wegrzyn, and J. M. Soos. 1993. Staphylococcal enterotoxins can reactivate experimental allergic encephalomyelitis. *Proc. Natl. Acad. Sci. USA 90:8543.*
5. Matthews, W. B., A. Compston, I. V. Allen, and C. N. Martyn. 1991. *McAlpine's Multiple Sclerosis.* Edinburgh, Churchill Livingstone.
6. Fritz, R. B., J. C.-H. Chou, and D. E. McFarlin. 1983. Relapsing murine experimental allergic encephalomyelitis induced by myelin basic protein. *J. Immunol. 130:1024.*
7. Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P. H. Krammer, and H. Wagner. 1992. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. Exp. Med. 175: 191.*
8. Morio, T., R. Geha, and T. Chatila. 1994. Engagement of MHC class II molecules by staphylococcal superantigens activates src-type protein tyrosine kinases. *Eur. J. Immunol. 24:651.*
9. Mourad, W., K. Mehindate, T. J. Schall, and S. R. McColl. 1992. Engagement of major histocompatibility complex class II molecules by superantigen induces inflammatory cytokine gene expression in human fibroblast-like synoviocytes. *J. Exp. Med. 175:613.*
10. Pontzer, C. H., N. D. Griggs, and H. M. Johnson. 1993. Agonist properties of a microbial superantigen peptide. *Biochem. Biophys. Res. Commun. 193:1191.*
11. Hauschildt, S., W. Bessler, and P. Scheipers. 1993. Engagement of major histocompatibility complex class II molecules leads to nitrite production in bone marrow-derived macrophages. *Eur. J. Immunol. 23:2988.*
12. Schmitz, J., and A. Radbruch. 1992. Distinct antigen presenting cell-derived signals induce Th cell proliferation and expression of effector cytokines. *Int. Immunol. 4:43.*
13. Cardell, S., I. Hoiden, and G. Moller. 1993. Manipulation of the superantigen-induced lymphokine response: selective induction of interleukin-10 or interferon-γ synthesis in small resting CD4$^+$ T cells. *Eur. J. Immunol. 23:523.*
14. Nagelkerken, L., K. J. Gollob, M. Tielemans, and R. L. Coffman. 1993. Role of transforming growth factor-β in the preferential induction of T helper cells of type 1 by staphylococcal enterotoxin B. *Eur. J. Immunol. 23:2301.*
15. Krakauer, T. 1994. Costimulatory receptors for the superantigen staphylococcal enterotoxin B on human vascular endothelial cells and T cells. *J. Leukocyte Biol. 56:458.*
16. Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biological effects on human lymphocytes. *J. Exp. Med. 170:827.*
17. Stern, A. S., F. J. Podlaski, J. D. Hulmes, Y. E. Pan, P. M. Quinn, A. G. Wolitzky, P. C. Familletti, D. L. Stremlo, T. Truitt, R. Chizzonite, and M. K. Gately. 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA 87:6808.*
18. Scott, P. 1993. IL-12: initiation cytokine for cell-mediated immunity. *Science 260:496.*
19. Trinchieri, G. 1993. Interleukin-12 and its role in the generation of Th1 cells. *Immunol. Today 14:335.*
20. Leonard, J. P., K. E. Waldburger, and S. J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med. 181:381.*
21. Waldburger, K. E., R. C. Hastings, R. G. Schaub, S. J. Goldman, and J. P. Leonard. 1996. Adoptive transfer of experimental allergic encephalomyelitis after in vitro treatment with recombinant murine interleukin-12. *Am. J. Pathol. 148:375.*
22. Smith, T., A. K. Hewson, C. I. Kingsley, J. P. Leonard, and M. L. Cuzner. 1997. Interleukin-12 induces relapse in experimental allergic encephalomyelitis in the Lewis rat. *Am. J. Pathol. 150:1909.*
23. Leung, D. Y. M., M. Gately, A. Trumble, B. Fergusson-Darnell, P. M. Schlievert, and L. J. Picker. 1995. Bacterial superantigens induce T cell expression of the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen, via stimulation of interleukin 12 production. *J. Exp. Med. 181:747.*
24. Skeen, M. J., M. A. Miller, T. M. Shinnick, and H. K. Ziegler. 1996. Regulation of murine macrophage IL-12 production: activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. *J. Immunol. 156:1196.*
25. Sriskandan, S., T. J. Evans, and J. Cohen. 1996. Bacterial superantigen-induced human lymphocyte responses are nitric oxide dependent and mediated by IL-12 and IFN-γ. *J. Immunol. 156:2430.*
26. D'Andrea, A., M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste-Amezaga, S. H. Chan, M. Kobayashi, D. Young, E. Nickbarg, et al. 1992. Production of natural killer cell stimulatory factor (NKSF/IL-12) by peripheral blood mononuclear cells. *J. Exp. Med. 176:1387.*
27. Segal, B. M., D. M. Klinman, E. M. Shevach. 1997. Microbial products induce autoimmune disease by an IL-12-dependent pathway. *J. Immunol. 158:5087.*
28. Kuge, S., Y. Miura, Y. Nakamura, T. Mitomi, S. Habu, and T. Nishimura. 1995. Superantigen-induced human CD4$^+$ helper/killer T cell phenomenon: selective induction of Th1 helper/killer cells and application to tumor immunotherapy. *J. Immunol. 154:1777.*
29. Deibler, G. E., R. E. Martenson, and M. W. Kies. 1972. Large scale preparation of myelin basic protein from central nervous tissue of several mammalian species. *Prep. Biochem. 2:139.*
30. Wysocka, M., M. Kubin, L. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri. 1995. Interleukin-12 is required for interferon-γ production and lethality in lipopolysaccharide-induced shock in mice. *Eur. J. Immunol. 25:672.*
31. Gombold, J. L., R. M. Sutherland, E. Lavi, Y. Paterson, S. R. Weiss. 1995. Mouse hepatitis virus A-59-induced demyelination can occur in the absence of CD8$^+$ T cells. *Microb. Pathol. 18:211.*
32. Miethke, T., C. Wahl, K. Heeg, and H. Wagner. 1995. Superantigens: the paradox of T-cell activation versus inactivation. *Int. Arch. Allergy Immunol. 106:3.*
33. D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J. Exp. Med. 178:1041.*
34. Tripp, C. S., S. F. Wolff, and E. R. Unanue. 1993. Interleukin 12 and tumor necrosis factor are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA 90:3725.*
35. Trembleau, S., T. Germann, and M. K. Gately. 1995. The role of IL-12 in the induction of organ-specific autoimmune diseases. *Immunol. Today 16:383.*
36. Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri, and A. Sher. 1994. Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii. J. Immunol. 153:2533.*
37. Tripp, C. S., O. Kanagawa, and E. R. Unanue. 1995. Secondary response to *Listeria* infection requires IFN-γ but is independent of IL-12. *J. Immunol. 155: 3427.*

38. Neurath, M. F., I. Fuss, B. L. Kelsall, E. Stüber, and W. Strober. 1995. Antibodies to interleukin-12 abrogate established experimental colitis in mice. *J. Exp. Med. 182:1281.*
39. Joosten, L. A. B., E. Lubberts, M. M. A. Hellsen, and W. B. van den Berg. 1997. Dual role of IL-12 in early and late stages of murine collagen type II arthritis. *J. Immunol. 159:4092.*
40. Seder, R. A., B. Kelsall, and D. Jankovic. 1996. Differential roles for IL-12 in the maintenance of immune responses in infectious versus autoimmune disease. *J. Immunol. 157:2745.*
41. Peritt, D., M. Aste-Amezaga, F. Gerosa, C. Paganin, and G. Trinchieri. 1996. Interleukin-10 induction by IL-12: a possible modulatory mechanism? *Ann. NY Acad. Sci. 795:387.*
42. Winghagen, A., D. E. Anderson, A. Carrizosa, R. E. Williams, and D.A. Hafler. 1997. IL-12 induces human T cells secreting IL-10 with IFN-γ. *J. Immunol. 157:1127.*
43. Kennedy, M.K., D. S. Torrance, K. S. Picha, and K. M. Mohler. 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J. Immunol. 149:2496.*
44. Rott, O., B. Fleisher, and E. Cash. 1994. Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *Eur. J. Immunol. 24:1434.*
45. Cannella, B., Y. L. Gao, C. Brosnan, C. S. Raine. 1996. IL-10 fails to abrogate experimental autoimmune encephalomyelitis. *J. Neurosci. Res. 45:735.*
46. Crisi, G. M., L. Santambrogio, G. M. Hochwald, S. R. Smith, J. A. Carlino, and G. J. Thorbecke, 1995. *Staphylococcus* enterotoxin B and tumor-necrosis factor-α-induced relapses of experimental allergic encephalomyelitis: protection by transforming growth factor-β and interleukin-10. *Eur. J. Immunol. 25:3035.*
47. Segal, B. M., B. K. Dwyer, and E. M. Schevach. 1998. An Interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. *J. Exp. Med. 187:537.*
48. Lehmann, P. V., E. E. Sercarz, T. Forsthuber, C. M. Dayan, and G. Gammon. 1993. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol. Today 14:203.*
49. McRae, B. L., C. L. Vanderlugt, M. C. Dal Canto, and S. D. Miller. 1995. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J. Exp. Med. 182:75.*
50. Yu, M., J. M. Johnson, and V. K. Tuohy. 1996. Generation of autonomously pathogenic neo-autoreactive Th1 cells during the development of the determinant spreading cascade in murine autoimmune encephalomyelitis. *J. Neurosci. Res. 45:463.*
51. Kubin, M., M. Kamoun, and G. Trinchieri. 1994. Interleukin-12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J. Exp. Med. 180:211.*
52. Murphy, E., G. Terres, S. Macatonia, C.-S. Hsieh, J. Mattson, L. Lanier, M. Wysocka, G. Trinchieri, K. Murphy, and A. O'Garra. 1994. B7 and interleukin-12 cooperate for proliferation and interferon-γ production by mouse T helper clones that are unresponsive to B7 costimulation. *J. Exp. Med. 180:223.*
53. Miller, S. D., C. L. Vanderlugt, D. J. Lenschow, J. G. Pope, N. J. Karandikar, M. C. Dal Canto, and J. A. Bluestone. 1995. Blockade of CD28/B7 interaction prevents epitope spreading and clinical relapses of murine EAE. *Immunity 3:739.*
54. Perrin, P. J., D. Scott, L. Quigley, P. S. Albert, O. Feder, G. S. Gray, R. Abe, C. H. June, and M. K. Racke. 1995. Role of B7:CD28/CTLA4 in the induction of chronic relapsing experimental allergic encephalomyelitis. *J. Immunol. 154:1481.*
55. Miethke, T., C. Wahl, D. Regele, H. Gaus, K. Heeg, and H. Wagner. 1993. Superantigen-mediated shock: a cytokine release syndrome. *Immunobiology 189:270.*
56. Ogawa, M., T. Tsutsui, J.-P. Zou, R. Wijesuriya, W.-G. Yu, S. Herrmann, T. Kubo, H. Fujiwara, and T. Hamaoka. 1997. Enhanced induction of very late antigen 4/lymphocyte function-associated antigen 1-dependent T-cell migration to tumor sites following administration of interleukin-12. *Cancer Res. 57:2216.*
57. Baron, J. L., J. A. Madri, N. H. Ruddle, G. Hashim, C. A. Janeway. 1993. Surface expression of α4 integrin by $CD4^+$ T cells is required for their entry into brain parenchyma. *J. Exp. Med. 177:57.*
58. Marth, T., W. Strober, and B. L. Kelsall. 1996. High dose oral tolerance in ovalbumin TCR-transgenic mice: systemic neutralization of IL-12 augments TGF-β secretion and T cell apoptosis. *J. Immunol. 157:2348.*
59. Brocke, S., K. Gijbels, M. Allegretta, I. Ferber, C. Piercy, T. Blankenstein, R. Martin, U. Utz, N. Karin, D. Mitchell, et al. 1996. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature 379:343.*
60. Kuruvilla, A. P., R. Shah, G. M. Hochwald, M. Liggitt, A. Palladino, and G. J. Thorbecke. 1991. Protective effect of transforming growth factor β1 on experimental autoimmune diseases in mice. *Proc. Natl. Acad. Sci. USA 88:2918.*
61. Racke, M. K., S. Dhib-Jalbut, B. Cannella, P. Albert, C. S. Raine, and D. E. McFarlin. 1991. Prevention and treatment of chronic relapsing experimental allergic encephalomyelitis by transforming growth factor-β1. *J. Immunol. 146:3012.*
62. Soos, J. M., P. S. Subramaniam, A. C. Hobeika, J. Schiffenbauer, and H. M. Johnson. 1995. The IFN pregnancy recognition hormone IFN-γ blocks both development and superantigen reactivation of experimental allergic encephalomyelitis without associated toxicity. *J. Immunol. 155:2747.*
63. Santambrogio, L., G. M. Hochwald, B. Saxena, C.-H. Leu, J. E. Martz, J. A. Carlino, N. H. Ruddle, M. A. Palladino, L. I. Gold, and G. J. Thorbecke. 1993. Studies on the mechanism by which transforming growth factor-β (TGF-β) protects against allergic encephalomyelitis: antagonism between TGF-β and tumor necrosis factor. *J. Immunol. 151:1116.*
64. Baker, D., D. Butler, B. J. Scallon, J. K. O'Neill, and J. L. Turk. 1994. Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. *Eur. J. Immunol. 24:2040.*
65. Hunter, C., L. Bermudez, H. Beernink, W. Waegell, and J. S. Remington. 1995. Transforming growth factor-β inhibits interleukin-12-induced production of interferon-γ by natural killer cells: a role for transforming growth factor-β in the regulation of T-cell independent resistance to *Toxoplasma gondii. Eur. J. Immunol. 25:994.*
66. Scharton-Kersten, T., L. C. C. Afonso, M. Wysocka, G. Trinchieri, and P. Scott. 1995. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. *J. Immunol. 154:5320.*
67. Goverman, J., A. Woods, L. Larson, L. P. Weiner, L. Hood, and D. M. Zaller. 1993. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell 72:551.*
68. Lichtman, S. N., J. Wang, R. B. Sartor, C. Zhang, D. Bender, F. G. Dalldorf, and J. H. Schwab. 1995. Reactivation of arthritis induced by small bowel bacterial overgrowth in rats: role of cytokines, bacteria, and bacterial polymers. *Infect. Immun. 63:2295.*
69. C. S. Constantinescu, B. D. Hondowicz, M. M. Ellosa, M. Wysocka, G. Trinchieri, and P. Scott. 1998. The role of IL-12 in the maintenance of an established Th1 response in experimental leishmaniasis. *Eur. J. Immunol. 28:2227.*

ORIGINAL ARTICLE

Anti–Interleukin-12 Antibody for Active Crohn's Disease

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ABSTRACT

BACKGROUND

Crohn's disease is associated with excess cytokine activity mediated by type 1 helper T (Th1) cells. Interleukin-12 is a key cytokine that initiates Th1-mediated inflammatory responses.

METHODS

This double-blind trial evaluated the safety and efficacy of a human monoclonal antibody against interleukin-12 (anti–interleukin-12) in 79 patients with active Crohn's disease. Patients were randomly assigned to receive seven weekly subcutaneous injections of 1 mg or 3 mg of anti–interleukin-12 per kilogram of body weight or placebo, with either a four-week interval between the first and second injection (Cohort 1) or no interruption between the two injections (Cohort 2). Safety was the primary end point, and the rates of clinical response (defined by a reduction in the score for the Crohn's Disease Activity Index [CDAI] of at least 100 points) and remission (defined by a CDAI score of 150 or less) were secondary end points.

RESULTS

Seven weeks of uninterrupted treatment with 3 mg of anti–interleukin-12 per kilogram resulted in higher response rates than did placebo administration (75 percent vs. 25 percent, P=0.03). At 18 weeks of follow-up, the difference in response rates was no longer significant (69 percent vs. 25 percent, P=0.08). Differences in remission rates between the group given 3 mg of anti–interleukin-12 per kilogram and the placebo group in Cohort 2 were not significant at either the end of treatment or the end of follow-up (38 percent and 0 percent, respectively, at both times; P=0.07). There were no significant differences in response rates among the groups in Cohort 1. The rates of adverse events among patients receiving anti–interleukin-12 were similar to those among patients given placebo, except for a higher rate of local reactions at injection sites in the former group. Decreases in the secretion of interleukin-12, interferon-γ, and tumor necrosis factor α by mononuclear cells of the colonic lamina propria accompanied clinical improvement in patients receiving anti–interleukin-12.

CONCLUSIONS

Treatment with a monoclonal antibody against interleukin-12 may induce clinical responses and remissions in patients with active Crohn's disease. This treatment is associated with decreases in Th1-mediated inflammatory cytokines at the site of disease.

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INTERLEUKIN-12 IS A KEY CYTOKINE THAT drives the inflammatory response mediated by type 1 helper T (Th1) cells.[1,2] As such, it underlies both normal host responses to a variety of intracellular bacterial, fungal, and protozoal pathogens and the abnormal inflammatory responses that accompany many autoimmune diseases, such as Crohn's disease. Crohn's disease is characterized by increased production of interleukin-12 by antigen-presenting cells in intestinal tissue and interferon-γ and tumor necrosis factor α (TNF-α) by intestinal lymphocytes and macrophages.[3-7] These inflammatory cytokines induce and sustain the granulomatous inflammation and bowel-wall thickening that are hallmarks of Crohn's disease.

Targeting interleukin-12 with antibodies is an effective treatment for the intestinal inflammation in animal models of Crohn's disease. Mice with trinitrobenzene sulfonate–induced colitis have a Th1-mediated gut inflammation characterized by greatly increased production of interleukin-12, interferon-γ, and TNF-α. In mice, administration of a monoclonal antibody against interleukin-12 (anti–interleukin-12) can result in the resolution of established colitis and, if given at the time of induction of colitis, can prevent inflammation.[8] Anti–interleukin-12 can also prevent and treat the spontaneous colitis seen in models of Th1-mediated inflammation such as mice that overexpress the human CD3ϵ gene and mice deficient in interleukin-10.[9,10]

We conducted a multisite, randomized, double-blind, placebo-controlled study to evaluate the safety and efficacy of anti–interleukin-12 for Crohn's disease. We determined the rates of remission, clinical response, and adverse events using two doses and two dosing schedules and measured changes in the secretion of cytokines by mononuclear cells of the colonic lamina propria (LPMCs) after anti–interleukin-12 treatment.

METHODS

PATIENTS

Eligible male or female patients were at least 18 years old, had received a diagnosis of Crohn's disease, and had a score on the Crohn's Disease Activity Index (CDAI) of 220 to 450 within two weeks before beginning treatment (CDAI scores can range from 0 to 600, with higher scores indicating more severe disease).[11] Eligible patients could continue to take concomitant medications if such therapy had begun at least 2 weeks before study treatment in the case of antibiotics; at least 4 weeks before in the case of mesalamine, sulfasalazine, prednisone (20 mg per day or less), or prednisone equivalent; and at least 12 weeks before in the case of azathioprine or mercaptopurine. The doses of these medications had to remain stable throughout the treatment period. Patients who had received antibody against TNF-α, methotrexate, cyclosporine, tacrolimus, thalidomide, or mycophenolate mofetil within four months before randomization were excluded, as were patients who had received any experimental agent or more than 20 mg of prednisone or prednisone equivalent per day within four weeks before randomization and patients who had received corticosteroid or mesalamine enemas within seven days or nonsteroidal antiinflammatory drugs within 24 hours before randomization. Female patients were required to use two forms of contraception throughout the study period. Other exclusion criteria included the presence of an ostomy, intestinal resection resulting in the short-bowel syndrome, a clinically significant abnormality on chest x-ray film or electrocardiogram, bowel obstruction or a known high-grade stricture, probable requirement for intestinal surgery within 12 weeks after randomization, stool examination or culture positive for pathogens or *Clostridium difficile* toxin, Cushing's syndrome, active acute infection requiring antibiotics, clinically significant laboratory abnormalities, active hepatitis B or C virus infection, seropositivity for the human immunodeficiency virus, a history of cancer, and a history of anaphylactic reaction to anti–TNF-α therapy. Women who were pregnant or breast-feeding were excluded. Patients were also excluded if they had a history of tuberculosis, positive purified protein derivative test, receipt of bacille Calmette–Guérin vaccine, or moderate or severe persistent asthma.

Patients were screened for eligibility at participating sites after the protocol had been approved by local institutional review boards or ethics committees. Eligible patients were randomly assigned to receive treatment at 15 centers in the United States, Germany, and the Netherlands from October 2000 to January 2002.

STUDY DESIGN

The study was a multicenter, randomized, placebo-controlled, double-blind, phase 2 clinical trial. After providing written informed consent, patients entered a 14-day screening phase to determine eligibility and pretreatment measurements. Two cohorts

were then enrolled sequentially: Cohort 1 received one injection followed four weeks later by one injection per week for six weeks, and Cohort 2 received one injection per week for seven weeks. On the basis of pharmacokinetic and preclinical data showing that 1 μg of anti–interleukin-12 per milliliter of serum blocked 90 percent of interleukin-12–induced neopterin release in vivo, an equivalent dose of 1 mg per kilogram of body weight was chosen as the lower-limit dose. The four-week interval between the first and second dose in Cohort 1 was chosen to assess the safety of a single dose of anti–interleukin-12 in patients with Crohn's disease. Patients were randomly assigned to receive subcutaneous placebo, anti–interleukin-12 at a dose of 1 mg per kilogram, or anti–interleukin-12 at a dose of 3 mg per kilogram in a 1:2:2 ratio by means of an independent, computer-generated randomization schedule without stratification or block allocation. Patients in Cohort 1 were seen two weeks after the first injection and at weekly intervals coinciding with the next six injections; patients in Cohort 2 were seen weekly during the seven-week treatment phase. All patients were followed for 18 weeks after the final injection of study drug and were seen at 6, 12, and 18 weeks.

The anti–interleukin-12 (ABT-874/J695, Wyeth Research and Abbott Laboratories) is a recombinant, exclusively human-sequence, full-length $IgG_1\lambda$ antibody genetically modified to recognize interleukin-12 p40 protein. The antibody was supplied as a lyophilized powder and reconstituted with water to yield an isotonic solution. The placebo was the same isotonic solution administered in a volume appropriate to the assigned dose.

Patients enrolled at the National Institutes of Health (NIH) study site underwent colonoscopy just before the first injection and 48 hours after the final injection of study drug. At these times, biopsy samples were obtained within the same gut regions only from areas with endoscopically apparent inflammation or ulcer borders; biopsy specimens were used for histologic analysis and for the preparation of LPMCs for cytokine measurements.

All NIH investigators had complete access to the study data for review and analysis. The authors analyzed the data and wrote the article. The study sponsors (Wyeth and Abbott Laboratories) underwrote the costs of the study and measured the serum levels of antidrug antibodies and anti–interleukin-12. The sponsors were not involved in the decision to publish the results.

SAFETY ASSESSMENT

The primary objective of the study was the safety of anti–interleukin-12 treatment in patients with Crohn's disease. Changes in clinical, biochemical, and hematologic variables were assessed on days 15, 29, 43, and 64 in Cohort 1 and on days 8, 22, and 43 in Cohort 2 during the treatment phase and at each visit during the follow-up phase. The severity and cause (study drug or procedure) of adverse events were determined.

EFFICACY ASSESSMENT

Secondary outcomes included measurement of the rates of response and remission at two prespecified points: at the end of treatment (day 64 for Cohort 1 and day 43 for Cohort 2) and the end of follow-up (week 18). Remission was defined by a CDAI score of 150 or less, and a clinical response by a decrease in the CDAI score of at least 100 points. The CDAI was measured before treatment; during the week preceding days 15, 29, 43, and 64 in Cohort 1 and days 8, 22, and 43 in Cohort 2; and 6, 12, and 18 weeks after the final dose of study drug. Changes in the pretreatment levels of cytokine secretion by LPMCs and in the histologic score were also assessed at the end of treatment in the patients enrolled at the NIH.

ANTIDRUG ANTIBODY ASSESSMENT

Antidrug antibodies were measured in patients before treatment; on days 15, 29, 43, and 64 in Cohort 1 and days 22 and 43 in Cohort 2; and at each follow-up visit. Serum samples (diluted 1:25 and 1:75) were incubated in enzyme-linked immunosorbent assay (ELISA) plates coated with anti–interleukin-12; bound antidrug antibodies were detected by means of biotinylated anti–interleukin-12 and horseradish peroxidase–streptavidin. The operational lower limit of detection of antidrug antibodies was a serum dilution of 1:25, and only samples positive for antidrug antibodies at both the 1:25 and 1:75 dilutions underwent further titering.

PREPARATION OF LPMCs AND MEASUREMENT OF CYTOKINE RELEASE

LPMCs were prepared according to a modification of a previously described procedure.[4] Cytokines were measured by ELISA from supernatants of cultured cells after stimulation with antibodies against CD2 or CD3 plus CD28 (T-cell stimulus) and *Staphylococcus aureus* Cowan I, interferon-γ, and CD40 ligand trimer (antigen-presenting–cell stimulus).[12]

STATISTICAL ANALYSIS

The determination of the sample size was based primarily on safety outcomes and the ability to observe adverse events. Given a group of 16 patients, all of whom received the same dose according to the same schedule, the power to observe at least one adverse event, assuming a true rate of adverse events of 5 percent, 10 percent, or 20 percent, was 0.56, 0.82, or 0.97, respectively.

The demographic characteristics of the groups and the rates of adverse events were compared by means of descriptive methods, and significant differences were identified by means of the t-test or Fisher's exact test (all tests were two-tailed). Efficacy evaluation was a secondary aim, and data from all patients who underwent randomization were analyzed according to the intention-to-treat principle. Fisher's exact test was used to compare the remission and response rates between the treatment and placebo groups at the prespecified points (the end of treatment and the end of the 18-week follow-up period) without adjustment for multiple comparisons. Changes in mean cytokine secretion before and after treatment were evaluated by the paired t-test.

RESULTS

DEMOGRAPHIC AND BASELINE CHARACTERISTICS OF THE PATIENTS

Among 123 patients who were screened, 79 eligible patients were enrolled. The CDAI scores indicated that they had moderately active disease despite the concurrent use of medications for Crohn's disease by 75 percent of patients. Eighty-four percent completed all seven study-drug injections, 87 percent completed at least six, and 66 percent completed the entire protocol, including the 18-week follow-up period (Fig. 1). Demographic characteristics were similar within the two cohorts except that the group given 1 mg of anti–interleukin-12 per kilogram in Cohort 1 had had Crohn's disease for a significantly longer time and had a higher CDAI score than the placebo group and was less likely to be taking 5-aminosalicylate drugs or any medication for Crohn's disease than the group given 3 mg of anti–interleukin-12 per kilogram (Table 1). In Cohort 2, patients in the group given 3 mg per kilogram were more likely than those in the placebo group to be taking concomitant immunomodulatory medications (Table 1).

SAFETY AND ADVERSE EVENTS

The most frequently reported adverse event was a local reaction at the injection site (Table 2). This event was significantly more common among patients who received either 1 mg or 3 mg of anti–interleukin-12 per kilogram (range, 77 to 88 percent) than among patients who received placebo (25 percent). The majority of injection reactions to anti–interleukin-12 were mild (88 percent), and all responded to symptomatic therapy. One patient withdrew from the study owing to a local reaction after each of the first two injections. The incidence of other adverse events that occurred in more than 10 percent of patients was not significantly different among the groups in either cohort (Table 2). There were no serious infections.

Four patients discontinued the study because of adverse events: two in the group given 1 mg of anti–interleukin-12 per kilogram (one had injection-site reactions, and one was given a diagnosis of a small-bowel dysplastic adenoma after receiving four injections) and two in the placebo group (one received a diagnosis of a peritoneal abscess, and one had an increase in the symptoms of Crohn's disease). Nine serious adverse events occurred, none attributed to anti–interleukin-12. Two of the nine serious adverse events occurred in patients in the placebo group. The other seven occurred in patients who received anti–interleukin-12: two had adverse events two to three months after the last dose of drug (one had diarrhea and dehydration and one had migraine and bone pain); one was hospitalized for partial obstruction of the small bowel, which did not recur despite continued administration of drug; two had adverse events related to preexisting neoplastic conditions (skin cancer and dysplastic tubular adenoma); one had substance abuse requiring an evaluation in the emergency room; and one patient's wife became pregnant during the study (a concern because of unknown teratogenic effects).

Clinically significant laboratory abnormalities were noted in 17 patients who received anti–interleukin-12 and 5 patients who received placebo. The most frequent abnormalities were hyperuricemia (uric acid levels were 7.5 to 10.0 mg per deciliter in nine patients who received anti–interleukin-12 and two who received placebo and exceeded 10 mg per deciliter in one patient given anti–interleukin-12), hypoglycemia (in two patients given anti–interleukin-12), hyperamylasemia (in two patients given anti–interleukin-12), and hyperphosphatemia (in

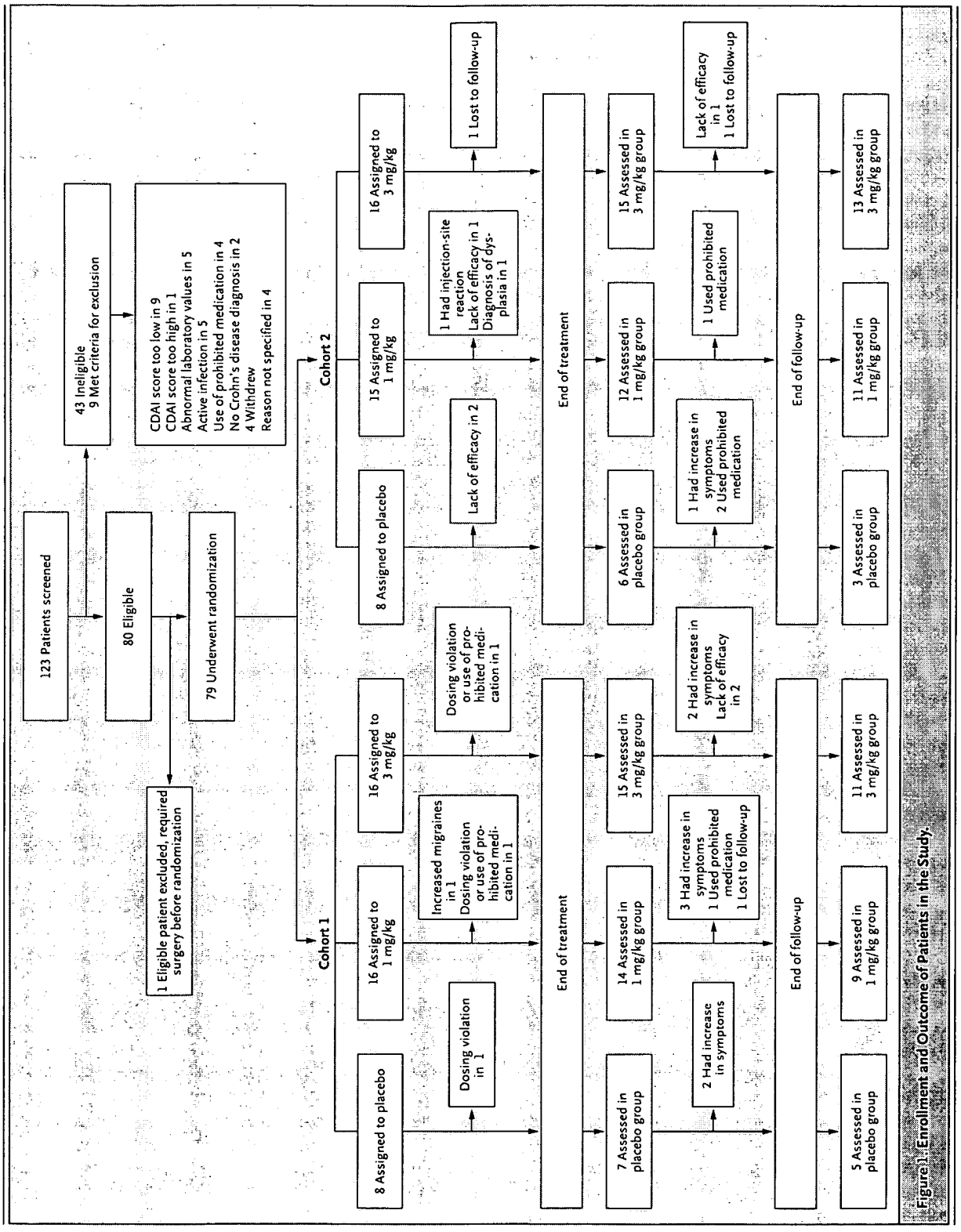

Figure 1. Enrollment and Outcome of Patients in the Study.

Table 1. Baseline Demographic and Clinical Characteristics.*

Characteristic	Cohort 1			Cohort 2		
	Placebo (N=8)	Anti–Interleukin-12 Antibody, 1 mg/kg (N=16)	Anti–Interleukin-12 Antibody, 3 mg/kg (N=16)	Placebo (N=8)	Anti–Interleukin-12 Antibody, 1 mg/kg (N=15)	Anti–Interleukin-12 Antibody, 3 mg/kg (N=16)
Age — yr						
Mean	36.9±13.3	43.1±12.6	42.5±14.2	41±12.6	39.9±13.9	39.8±13.4
Range	20–60	28–80	21–71	23–62	22–72	23–66
Sex — no. (%)						
Male	3 (38)	4 (25)	5 (31)	3 (38)	3 (20)	8 (50)
Female	5 (62)	12 (75)	11 (69)	5 (62)	12 (80)	8 (50)
Weight — kg						
Mean	71.9±11.9	68.8±13.1	68.5±14.5	89.1±25.7	72.5±27.4	67.3±14
Range	54–92	48–91	48–102	55–120	48–131	48–98
Duration of disease — mo						
Mean	76±58	165±145†	139±115	125±117	175±177	154±112
Range	8–181	34–518	15–362	5–305	6–623	12–396
Crohn's Disease Activity Index score‡						
Mean	279±53	360±54†	300±53	335±63	323±54	356±75
Range	231–399	282–469§	204–380¶	235–427	250–440	239–529§
Disease location — no. (%)						
Ileum	4 (50)	3 (19)	4 (25)	4 (50)	4 (27)	3 (19)
Colon	3 (38)	5 (31)	6 (38)	1 (12)	2 (13)	7 (44)
Ileum and colon	1 (12)	8 (50)	6 (38)	3 (38)	9 (60)	6 (38)
Fistulizing disease — no. (%)	3 (38)	10 (62)	7 (44)	3 (38)	6 (40)	10 (62)
Prior bowel resection — no. (%)	4 (50)	4 (25)	4 (25)	3 (38)	5 (33)	7 (44)
Concomitant medication — no. (%)						
None for Crohn's disease	2 (25)	7 (44)	1 (6)‖	4 (50)	4 (27)	2 (12)
5-Aminosalicylate drugs	6 (75)	7 (44)	14 (88)‖	3 (38)	7 (47)	10 (62)
Corticosteroids	4 (50)	3 (19)	4 (25)	2 (25)	6 (40)	3 (19)
Immunomodulators	2 (25)	5 (31)	7 (44)	0	4 (27)	8 (50)†

* Plus–minus values are means ±SD.
† P<0.05 for the comparison with the placebo group in the cohort.
‡ Scores for the Crohn's Disease Activity Index can range from 0 to 600, with higher scores indicating more severe disease.
§ Two patients had a baseline score of more than 450, and both were included in the analysis.
¶ One patient had a baseline score of less than 220 and was included in the analysis.
‖ P<0.05 for the comparison with the group given 1 mg per kilogram in the cohort.

two patients given placebo). None of these abnormalities required withdrawal from the study.

DEVELOPMENT OF ANTIDRUG ANTIBODIES

Antidrug antibodies were detected in only three patients, all of whom received 1 mg of anti–interleukin-12 per kilogram. Antidrug antibodies developed three to four months after the final injection in two patients in Cohort 1 who had a response. These two patients also had unexpectedly low serum levels of anti–interleukin-12 and early clearance of anti–interleukin-12 from the serum; the

Table 2. Adverse Events Occurring in More Than 10 Percent of Patients and Incidence of Antidrug Antibodies against Anti–Interleukin-12.

Adverse Event	Cohort 1			Cohort 2		
	Placebo (N=8)	Anti–Interleukin-12 Antibody, 1 mg/kg (N=16)	Anti–Interleukin-12 Antibody, 3 mg/kg (N=16)	Placebo (N=8)	Anti–Interleukin-12 Antibody, 1 mg/kg (N=15)	Anti–Interleukin-12 Antibody, 3 mg/kg (N=16)
	number of patients (percent)					
Nausea	2 (25)	3 (19)	2 (12)	1 (12)	0	3 (19)
Vomiting	0	3 (19)	2 (12)	1 (12)	0	3 (19)
Abdominal pain	0	1 (6)	1 (6)	0	1 (7)	2 (12)
Arthralgia	0	0	2 (12)	1 (12)	3 (20)	1 (6)
Urinary tract infection	0	3 (19)	1 (6)	1 (12)	1 (7)	0
Bronchitis	0	0	2 (12)	0	0	2 (12)
Cough	2 (25)	2 (12)	1 (6)	1 (12)	0	0
Headache	1 (12)	6 (38)	1 (6)	1 (12)	1 (7)	5 (31)
Fever	1 (12)	4 (25)	4 (25)	2(25)	1 (7)	3 (19)
Fatigue	0	2 (12)	1 (6)	2 (25)	1 (7)	2 (12)
Local injection-site reaction	3 (38)	12 (75)	13 (81)	1 (12)	12 (80)*	15 (94)*
Antidrug antibodies against anti–interleukin-12†	0	2 (12)	0	0	1 (7)	0

* P<0.005 for the comparison with the placebo group in the cohort.
† Values reflect the overall incidence of antidrug antibodies.

loss of response coincided with the detection of antidrug antibodies in one patient and preceded the detection of antidrug antibodies by two months in the other patient. Antidrug antibodies were detected before treatment in one patient in Cohort 2 and persisted to the end of follow-up. This finding was not considered to be drug related, because it was present before treatment, there was no change in titer with treatment, and there was no association with low serum levels of anti–interleukin-12. Since the presence of measurable levels of anti–interleukin-12 in serum can interfere with the detection of antidrug antibodies, this assay may have underestimated the incidence of antidrug antibodies. However, only three additional patients who received anti–interleukin-12 had unexpectedly low serum antibody levels, suggesting the presence of antidrug antibodies, but no antidrug antibodies were detected, even when anti–interleukin-12 levels fell below quantifiable levels.

CLINICAL RESPONSE AND REMISSION

Cohort 1

The Cohort 1 dosing schedule gave us an opportunity to assess the effect of a single dose of anti–interleukin-12 as compared with that of placebo. Four weeks after one injection of study drug, 50 percent of the patients in the group given 1 mg per kilogram had a clinical response, as compared with 19 percent in the group given 3 mg per kilogram and 13 percent in the placebo group (Fig. 2A). The rate of remission at this time was 19 percent in the group given 1 mg per kilogram, 12 percent in the group given 3 mg per kilogram, and 0 percent in the placebo group (Fig. 2C). However, at the time of the final injection (nine weeks), the response rate was 63 percent in the group given 1 mg per kilogram, 56 percent in the group given 3 mg per kilogram, and 38 percent in the placebo group, and the remission rates were 31 percent, 44 percent, and 38 percent, respectively. At the end of the 18-week follow-up phase, the group given 3 mg per kilogram maintained remission and response rates of 50 percent, as compared with 19 percent each in the group given 1 mg per kilogram and 13 percent and 25 percent, respectively, in the placebo group. At no time were the rates in the anti–interleukin-12 groups significantly different from those in the placebo group.

Cohort 2

The Cohort 2 dosing schedule was a continuous induction therapy of seven weekly injections of study drug. At the time of the final injection (seven

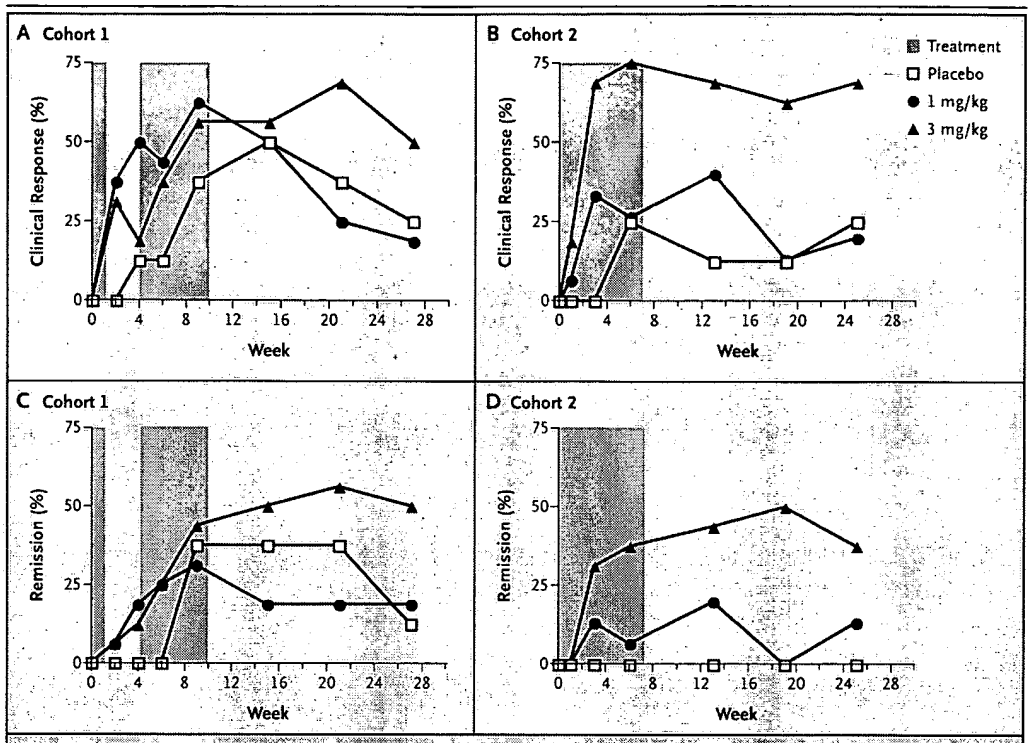

Figure 2. Rates of Clinical Response (Panels A and B) and Remission (Panels C and D) among Patients with Active Crohn's Disease Receiving Anti–Interleukin-12 or Placebo.
Shaded areas mark seven-day periods after injections.

weeks), the respective response and remission rates were 27 percent and 8 percent in the group given 1 mg per kilogram, 75 percent and 38 percent in the group given 3 mg per kilogram, and 25 percent and 0 percent in the placebo group (Fig. 2B and 2D). At the end of the 18-week follow-up phase, the respective response and remission rates were 69 percent and 38 percent in the group given 3 mg per kilogram, 20 percent and 13 percent in the group given 1 mg per kilogram, and 25 percent and 0 percent in the placebo group. The response rate was significantly higher in the group given 3 mg per kilogram than in the placebo group at the end of treatment (75 percent vs. 25 percent, P=0.03) but not at the end of follow-up (69 percent vs. 25 percent, P=0.08). Remission rates in the group given 3 mg per kilogram did not differ significantly from those in the placebo group at either the end of treatment or the end of follow-up (38 percent at both times vs. 0 percent at both times, P=0.07).

EFFECTS OF ANTI–INTERLEUKIN-12 ON CYTOKINE SECRETION BY LPMCs AND HISTOLOGIC FINDINGS

Treatment with anti–interleukin-12 was associated with decreases in the secretion of interleukin-12, interferon-γ, and TNF-α by LPMCs after in vitro T-cell stimulation (used for interferon-γ and TNF-α) and antigen-presenting–cell stimulation (used for interleukin-12) (Fig. 3). Although the secretion of interleukin-6 also decreased after antibody treatment ($P<0.06$), the changes in the secretion of interleukin-10 and interleukin-18 were not significant. The magnitude of change in cytokine secretion did not correlate with the dose or regimen of anti–interleukin-12, but the only antibody-treated patient in this subgroup who did not have a clinical response (Patient 8 in Fig. 3) had an increase in interferon-γ secretion and no change in TNF-α secretion. One patient in the subgroup (Patient 2) (data not shown) was randomly assigned to receive placebo but in-

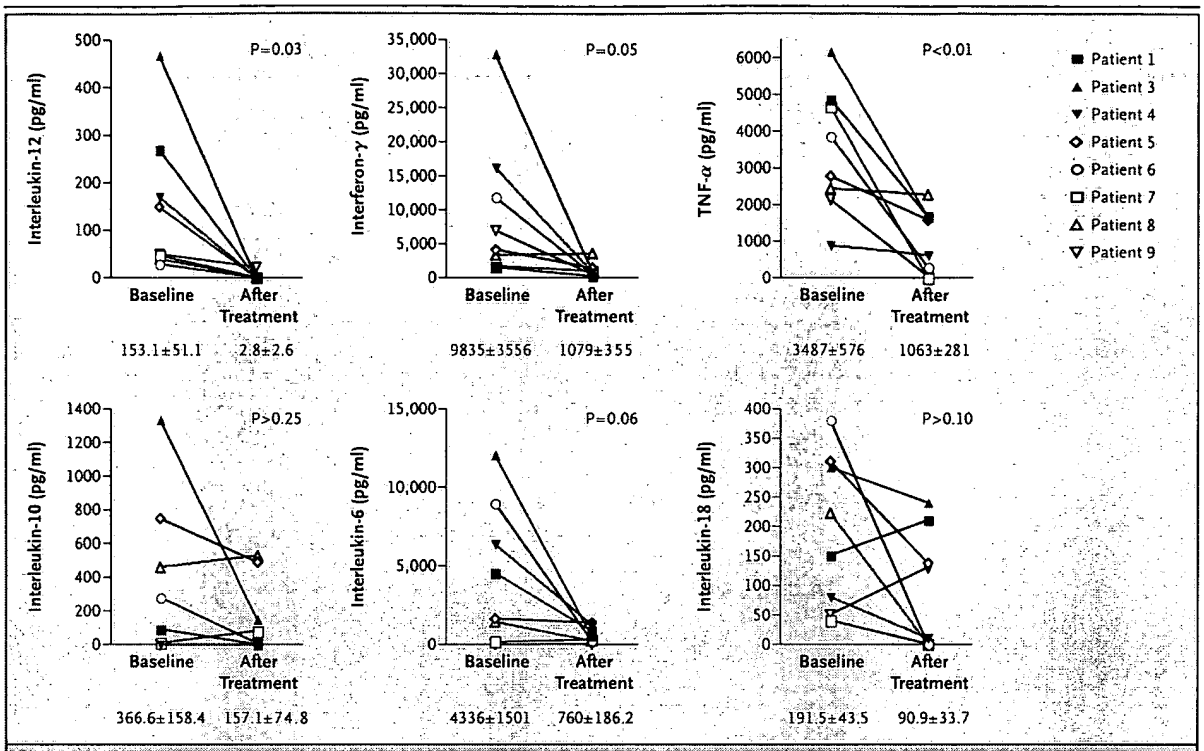

Figure 3. Cytokine Secretion by Mononuclear Cells of the Colonic Lamina Propria from Patients with Crohn's Disease before and after Treatment with Anti–Interleukin-12.

The mean (±SD) levels of each cytokine before and after treatment are shown under the x-axis. Solid symbols indicate patients in Cohort 1, and open symbols patients in Cohort 2.

advertently received a single dose of antibody as the fifth scheduled dose. This patient's baseline secretion of interleukin-12 (310 pg per milliliter), interferon-γ (18,515 pg per milliliter), and TNF-α (6468 pg per milliliter) fell to 0, 1471, and 4342 pg per milliliter, respectively, two weeks later, coinciding with the induction of remission (reflected by a decrease in the CDAI score from 279 to 143).

Treatment with anti–interleukin-12 in this subgroup of patients was also associated with a decrease in mucosal histologic abnormalities. Of the eight patients in the subgroup who received anti–interleukin-12, seven had a response, and six of these seven had improved mucosal histologic scores. Using a modified d'Haens scoring system (no points were assigned for number of affected biopsy samples, so the maximal possible score was 13 instead of 16),[13] we found that the mean (±SD) score in these seven patients was 6.9±3.8 before treatment and 3.6±2.1 at the end of treatment (P=0.06). The one patient who did not have a response to antibody treatment and who had no decrease in cytokine secretion had an increase in the histologic score. The patient in the placebo group who received a single dose of anti–interleukin-12 two weeks before the end-of-treatment biopsies also had an increase in the histologic score; this change is consistent with the patient's worsening symptoms just before the single dose of antibody and notable in the light of the decrease in cytokine secretion by LPMCs two weeks later. In general, histologic improvement was characterized by decreased numbers of neutrophils, lymphocytes, and plasma cells and reduced epithelial damage, with the reappearance of goblet cells.

DISCUSSION

Our data demonstrate that targeting interleukin-12 p40 with a specific antibody may induce a clini-

cal response or remission in patients with active Crohn's disease. Furthermore, the resulting clinical responses and remissions could be rapid in onset and durable. Patients treated with 3 mg of anti–interleukin-12 per kilogram had clinical responses after three weekly injections, and the response was sustained for at least 18 weeks after treatment.

The uninterrupted series of weekly injections of 3 mg per kilogram (in Cohort 2) resulted in higher response rates than the interrupted series (in Cohort 1). The difference in treatment effects between the regimens may be related to the speed with which maximal serum levels of anti–interleukin-12 are attained or sustained. In Cohort 1, the mean anti–interleukin-12 serum level was 1264±1675 ng per milliliter before the injection at week 4, as compared with 8271±2523 ng per milliliter before the injection at week 3 in Cohort 2, whereas the respective end-of-treatment levels of anti–interleukin-12 were similar (10,022±5807 ng per milliliter in Cohort 1 and 9374±2898 ng per milliliter in Cohort 2). In addition, the response and remission rates in the placebo group in Cohort 2 were lower than those in the placebo group in Cohort 1. Because of the intention-to-treat analysis used, the curves for the placebo group in Cohort 1 included the remission in the patient who mistakenly received a single dose of 1 mg of anti–interleukin-12 per kilogram. On average, the placebo group in Cohort 2 had had Crohn's disease longer and had higher baseline CDAI scores than the placebo group in Cohort 1, differences that may have contributed to the lower remission rates (but possibly higher response rates[14]) in this group. The placebo group in Cohort 2 also had a lower rate of use of medications for Crohn's disease.

With the exception of local reactions at injection sites, there were no significant differences in the rate of adverse effects between placebo and anti–interleukin-12. For the most part these skin reactions were transient and mild and did not require treatment, and subcutaneous injection is a less costly and less time-consuming approach than intravenous infusion. Injection-site reactions occurred more frequently among patients who had a response to anti–interleukin-12 than among patients who did not have a response (44 of 52 [85 percent] vs. 7 of 11 [64 percent]), but the difference was not significant (P=0.20). Although the incidence of infections was not significantly increased in the anti–interleukin-12 group as a whole, the risk of infection with longer-term use remains to be established.

Evaluation of the cytokine responses before and soon after treatment showed that the secretion of interleukin-12, interferon-γ, and TNF-α by colonic LPMCs was notably decreased. Thus, targeting interleukin-12 can also reduce the levels of downstream effectors such as interferon-γ and TNF-α. However, it is important to note that since the anti–interleukin-12 that we used recognizes the interleukin-12 p40 chain, interleukin-23 — another Th1-inducing cytokine that shares the p40 chain — may also be involved in the observed responses.[15,16] The reduced secretion of interleukin-12 after treatment with anti–interleukin-12 suggests that treatment decreased the number of interleukin-12–producing macrophages[17] or that reduced secretion of interferon-γ eliminates the enhancing effect interferon-γ exerts on the secretion of interleukin-12 by macrophages.[18,19] In addition, response and remission rates did not appear to depend on increasing the secretion of interleukin-10, an antiinflammatory cytokine that is thought to play a role in regulating gut inflammation.[20]

The data from this early phase 2 study provide some evidence that an antibody targeted to interleukin-12 p40 is active against the inflammation of Crohn's disease as well as show that treatment with this agent may induce clinical response and remission. The clinical effects of anti–interleukin-12 in patients with Crohn's disease suggest that interleukin-12 has an important role in the ongoing inflammatory reaction of Crohn's disease, even in long-standing disease. Furthermore, these preliminary data will help guide the design of future studies to assess the efficacy of anti–interleukin-12 in Crohn's disease and, by extension, other Th1-mediated inflammatory disorders.

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Drs. Fuss, Neurath, and Strober report being coholders of a patent for the use of anti–interleukin-12 in Crohn's disease. Dr. Neurath reports having served as a speaker for Novartis. Dr. Mayer reports having served as a speaker for Centocor and a consultant for Therakos. Dr. Elson reports having served as a consultant for Corixa, Abbott, Solvay, and AstraZeneca and having received grant support from Sankyo. Dr. Sandborn reports having served as a consultant for Abbott, Ajinomoto Pharmaceuticals, Amgen, AstraZeneca, Atrix Laboratories, Berlex, Boehringer Ingelheim, Celltech, Centocor, Elan, Chemocentryx, Chugai, Combinatorx, Genentech, GlaxoSmithKline, H3 Pharma, Hoffmann–La Roche, Isis Pharmaceuticals, McNeil Consumer and Specialty, Merck, Millennium, Novartis, Ono Pharmaceuticals, Otsuka, Pharmadigm, Procter & Gamble, Prometheus, Protein Design Labs, Salix, Sangstat, Schering Canada, Serono, Shire, Targacept, Teva, Therakos, Tillott's Pharma, and Vela Pharmaceuticals; having served as a paid speaker for Abbott, AstraZene-

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APPENDIX

The members of the Anti–IL-12 Crohn's Disease Study Group are as follows (the numbers of patients enrolled at each institution are listed in parentheses): National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. (9) — P.J. Mannon, I.J. Fuss, W. Strober, and C. Groden; Mount Sinai School of Medicine, New York (10) — L. Mayer and D.H. Present; University of Alabama School of Medicine, Birmingham (5) — C.O. Elson; Mayo Clinic, Rochester, Minn. (5) — W.J. Sandborn; Mayo Clinic, Jacksonville, Fla. (5) — J. Cangemi; Cedars Sinai Medical Center, Los Angeles (4) — L. Kam; Pharma Research Group, Miami Beach, Fla. (6) — I. Bassan; Health Advance Institute, Peoria, Ill. (8) — B. Dolin; South Denver Gastroenterology, Englewood, Colo. (4) — L. Kim; Indianapolis Gastroenterology, Indianapolis (6) — S. Mahoney; Nashville Medical Research Institute, Nashville (5) — R. Pruitt; Advanced Clinical Research Institute, Anaheim, Calif. (6) — D. Riff; Medizinische Hochschule, Hannover, Germany (3) — M. Manns; Universitätsklinikum Eppendorf, Hamburg, Germany (2) — A. de Weerth; Academic Medical Center, Amsterdam (1) — S. van Deventer; and Wyeth Research, Cambridge, Mass. — U. Schwertschlag, C. Carini, N. Goodman, and M. Shapiro.

REFERENCES

1. Gately MK, Renzetti LM, Magram J, et al. The interleukin-12/interleukin-12 receptor system: role in normal and pathologic immune responses. Annu Rev Immunol 1998;16:495-521.
2. Trinchieri G. Proinflammatory and immunoregulatory functions of interleukin-12. Int Rev Immunol 1998;16:365-96.
3. Fais S, Capobianchi MR, Silvestri M, Mercuri F, Pallone F, Dianzani F. Interferon expression in Crohn's disease patients: increased interferon-γ and -α mRNA in the intestinal lamina propria mononuclear cells. J Interferon Res 1994;14:235-8.
4. Fuss IJ, Neurath M, Boirivant M, et al. Disparate $CD4^+$ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease: Crohn's disease LP cells manifest increased secretion of IFN-γ, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol 1996;157:1261-70.
5. Monteleone G, Biancone L, Marasco R, et al. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. Gastroenterology 1997;112:1169-78.
6. Parronchi P, Romagnani P, Annunziato F, et al. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. Am J Pathol 1997;150:823-32.
7. Plevy SE, Landers CJ, Prehn J, et al. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. J Immunol 1997;159:6276-82.
8. Neurath MF, Fuss I, Kelsall BL, Stuber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med 1995;182:1281-90.
9. Davidson NJ, Hudak SA, Lesley RE, Menon S, Leach MW, Rennick DM. IL-12, but not IFN-γ, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. J Immunol 1998;161:3143-9.
10. Simpson SJ, Shah S, Comiskey M, et al. T cell-mediated pathology in two models of experimental colitis depends predominantly on the interleukin-12/signal transducer and activator of transcription (Stat)-4 pathway, but is not conditional on interferon γ expression by T cells. J Exp Med 1998;187:1225-34.
11. Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index: National Cooperative Crohn's Disease Study. Gastroenterology 1976;70:439-44.
12. Jain A, Atkinson TP, Lipsky PE, Slater JE, Nelson DL, Strober W. Defects of T-cell effector function and post-thymic maturation in X-linked hyper-IgM syndrome. J Clin Invest 1999;103:1151-8.
13. D'Haens GR, Geboes K, Peeters M, Baert F, Penninckx F, Rutgeerts P. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. Gastroenterology 1998;114:262-7.
14. Su C, Lichtenstein GR, Krok K, Brensinger CM, Lewis JD. A meta-analysis of the placebo rates of remission and response in clinical trials of active Crohn's disease. Gastroenterology 2004;126:1257-69.
15. Frucht DM. IL-23: a cytokine that acts on memory T cells. Sci STKE 2002;2002(114):PE1.
16. Brombacher F, Kastelein RA, Alber G. Novel IL-12 family members shed light on the orchestration of Th1 responses. Trends Immunol 2003;24:207-12.
17. Fuss IJ, Marth T, Neurath MF, Pearlstein GR, Jain A, Strober W. Anti-interleukin 12 treatment regulates apoptosis of Th1 T cells in experimental colitis in mice. Gastroenterology 1999;117:1078-88.
18. Marth T, Kelsall BL. Regulation of interleukin-12 by complement receptor 3 signaling. J Exp Med 1997;185:1987-95.
19. Monteleone G, Parrello T, Monteleone I, Tammaro S, Luzza F, Pallone F. Interferon-gamma (IFN-gamma) and prostaglandin E2 (PGE2) regulate differently IL-12 production in human intestinal lamina propria mononuclear cells (LPMC). Clin Exp Immunol 1999;117:469-75.
20. Rennick DM, Fort MM. Lessons from genetically engineered animal models. XII. IL-10-deficient (IL)-10(−/−) mice and intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 2000;278:G829-G833.

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Treatment with Homodimeric Interleukin-12 (IL-12) p40 Protects Mice from IL-12-Dependent Shock but Not from Tumor Necrosis Factor Alpha-Dependent Shock

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The role of interleukin-12 (IL-12) was investigated in different shock models using anti-IL-12 reagents. IL-12 is composed of two disulfide-bonded subunits, p35 and p40. The IL-12 p40 homodimer $(p40)_2$ has been shown to be a potent IL-12 antagonist in vitro. We investigated its in vivo inhibitory capacity in different shock models of mice. We could demonstrate that $(p40)_2$ is able to protect mice from septic shock in primarily IL-12-dependent models such as the Shwartzman reaction and lipopolysaccharide (LPS)-induced shock, whereas $(p40)_2$ has no effect in the tumor necrosis factor alpha-dependent LPS/D-GalN shock model. In IL-12-dependent shock models, $(p40)_2$ inhibits IL-12-induced gamma interferon production and thereby interferes with the cascade of cytokine release, finally leading to death.

Septic shock results from uncontrolled sequential release of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, and gamma interferon (IFN-γ) in response to infection with gram-negative bacteria (1, 4, 5, 7, 23, 25, 26) and in response to endotoxins. Septic shock is of clinical relevance in spite of adequate antibiotic and supportive therapy.

In mice, several different lethal shock syndromes are known. The so-called Shwartzman reaction is induced by two consecutive injections of lipopolysaccharide (LPS) (3). A priming dose of LPS, injected intradermally in the footpad, is followed 24 h later by an intravenous challenge LPS injection. After this challenge injection that is not lethal without the priming injection, the mice die within 48 h from disseminated intravascular coagulation, vascular occlusion, hemorrhage, perivascular accumulation of leukocytes, and necrosis (24). This hypersensitivity reaction occurs only as a consequence of careful dosage and timing of LPS injections and needs specific routes of administration. The Shwartzman reaction is elicited by induced endogenous factors acting in a precise sequence. LPS induces the release of IL-12, which induces the production of IFN-γ, which finally primes macrophages (and other cell types). Upon LPS challenge, the lethal reaction is induced by a massive production of inflammatory cytokines by sensitized macrophages: TNF-α and IL-1 are thought to be the lethal effector molecules acting on target sites already sensitized by IFN-γ.

The second investigated shock model is induced by an intraperitoneal (i.p.) injection of an appropriate dose of LPS (LPS shock). In this model, it was reported that administration of polyclonal anti-IL-12 immunoglobulin G (IgG) substantially reduced LPS induction of IFN-γ production, but effects of anti-IL-12 on mortality were not described (13).

The third investigated shock model is induced by i.p. injection of LPS and D-galactosamine (LPS/D-GalN shock). It has been shown that D-GalN dramatically sensitizes mice to the lethal effect of LPS (8). Studies of TNF receptor (TNFR) p55-deficient mice revealed that lethality in this model depends on TNFR p55-mediated activation (20, 22). The effects of IL-12 blockade in this model have not been previously reported.

The purpose of this study was to characterize the role of IL-12 in shock induction in these various models. Furthermore, we were interested in testing whether IL-12 p40 homodimer $(p40)_2$, which has been shown in vitro to act as an IL-12 receptor antagonist (11, 16), is able to prevent IL-12-dependent activities in vivo. It was previously shown in both binding assays and bioassays in vitro that mouse IL-12 p40 dimer is 25- to 50-fold more potent as an IL-12 antagonist than mouse IL-12 p40 monomer (11). It could not be excluded that the lower levels of antagonist activity observed for preparations of purified p40 monomer were mediated, at least in part, by a small amount of contaminating dimer. In preliminary studies, the pharmacokinetics of mouse p40 dimer in mice appeared to be very similar to those of mouse IL-12 heterodimer, with a terminal elimination serum half-life of 3 to 5 h. Thus, in the present study, we focused on the potential in vivo antagonist activity of recombinant homodimeric IL-12 p40.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female NMRI and C57BL/6 mice, 8 to 10 weeks of age, were purchased from Biological Research Laboratory (Füllinsdorf, Switzerland).

Reagents. (i) LPS. *Serratia marcescens* LPS (Sigma Chemical Co., St. Louis, Mo.) and *Salmonella abortus-equi* LPS (SEBAK, Aidenbach, Germany) were dissolved in pyrogen-free saline to 10 mg/ml and sterilized by filtration through 0.22-μm-pore-size filters. Aliquots were stored frozen at −20°C.

(ii) IL-12 p40 homodimer. Dimeric p40 was produced by CHO cells stably transfected with mouse IL-12 p40 cDNA and purified as described previously (9). Purified p40 homodimer was >95% pure, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; endotoxin contamination was <10 IU/mg of p40 protein, as measured by the *Limulus* amebocyte lysate assay.

(iii) Antibodies. Rat monoclonal antibodies (MAbs) XMG1,2 and AN18 are IgG1 antibodies against mouse IFN-γ (mIFN-γ) (6, 21). Rat MAb GR20 is an IgG2a antibody specific for the mIFN-γ receptor (2). MAbs C15.6.7 and 10F6 neutralize mIL-12 activity (10, 27). Irrelevant rat IgG was from Sigma. All antibodies contained <0.05 IU/mg of protein, as detected by the *Limulus* amebocyte lysate assay. TNFR p55 IgG was produced and purified as described previously (14).

Shwartzman shock. The generalized Shwartzman reaction was elicited by two consecutive injections of *Serratia marcescens* LPS in female NMRI mice, 8 to 10

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TABLE 1. Homodimeric IL-12 p40 prevents mortality in the Shwartzman reaction

Treatment	No. of expt	No. of survivors/no. tested	% Survival
PBS	7	3/35	9
Anti-IFN-γ[a]	7	35/35	100[e]
Anti-IL-12[b]	1	4/5	80[e]
Rat IgG	2	0/10	0
$(p40)_2$[c]			
100 μg	4	19/20	95[e]
10 μg	2	2/10	20
1 μg	1	0/5	0
TNFR IgG[d]	2	2/10	20

[a] 10 μg of rat anti-mIFN-γ MAb XMG1,2 was administered i.p. at time of priming.

[b] 100 μg of rat anti-mIL-12 MAb 10F6 was administered i.p. at time of priming and 10 and 23 h later.

[c] Indicated doses of homodimeric p40 were administered i.p. at time of priming and 10 and 23 h later.

[d] 20 μg of TNFR IgG was administered i.p. at time of priming and 22 and 24 h later.

[e] Significantly different ($P < 0.002$) from values for PBS- or IgG-treated control groups as calculated by Fisher's exact test.

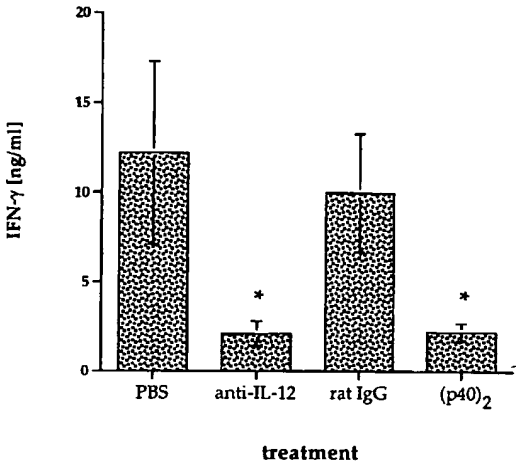

FIG. 1. Homodimeric p40 decreases IFN-γ serum levels in the Shwartzman reaction. Groups of five NMRI mice received two consecutive injections of LPS. For priming, 5 μg of LPS was given in the footpad, followed 24 h later by a challenge with 400 μg of LPS. Furthermore, mice were injected i.p. with 100 μg of anti-IL-12 MAb C15.6.7 (or irrelevant rat IgG) at time of priming or with 100 μg of $(p40)_2$ (or PBS) at time of priming and 10 and 23 h later. IFN-γ serum levels were determined 6 h after the challenge LPS injection. To facilitate IFN-γ determination, all mice were injected i.p. with MAb GR20 (200 μg) directed against the IFN-γ receptor at the time of LPS challenge. *, significantly different ($P < 0.001$) from values for PBS- or IgG-treated control groups as calculated by Student's t test.

weeks old (19). The priming injection was given in the footpad and was followed 24 h later by a challenge LPS injection given intravenously. The optimal doses of LPS were determined for each batch of LPS and ranged between 1 and 5 μg for the footpad injection and between 200 and 400 μg for the intravenous injection. Mortality was monitored after 24, 48, 72, and 96 h. No further mortality was seen after 96 h. In all experiments described, MAbs and IL-12 p40 homodimer were given i.p.

Endotoxin-induced shock. The LPS shock was induced in female C57BL/6 mice, 8 to 10 weeks old, by the i.p. injection of *S. abortus-equi* LPS (350 μg in 0.2 ml of sterile PBS per mouse). With a dose of 350 μg per mouse, the 100% lethal dose is almost reached (see Table 2). Mortality was monitored 24, 48, and 72 h after LPS injection.

LPS/D-GalN-induced shock. Female C57BL/6 mice, 8 to 10 weeks old, were injected i.p. with a mixture of *S. abortus-equi* LPS (0.1 μg in 0.2 ml of sterile PBS per mouse) and D-GalN (10 mg in 0.2 ml of sterile PBS per mouse; Roth, Karlsruhe, Germany) in order to sensitize to the lethal effects of LPS (8). Mortality was monitored after 24, 48, and 72 h.

Detection of serum IFN-γ levels. IFN-γ was measured by enzyme-linked immunosorbent assay using rat IgG1 MAbs AN18 and XMG1,2 (6, 21). The detection limit of this assay was 40 pg/ml.

RESULTS

Homodimeric p40 prevents mortality in the Shwartzman reaction. Groups of five NMRI mice received two consecutive injections of LPS. The priming injection was given in the footpad and was followed 24 h later by the intravenous challenge injection. The reaction was lethal only if the mice received both the preparative and the challenge injections. A single footpad or a single intravenous injection was ineffective in inducing shock.

It had been shown earlier that one single injection of MAb neutralizing mIFN-γ activity protects mice from lethality when given i.p. at time of priming (19). Similar results were obtained with MAb against mIL-12. Confirmatory data are presented in Table 1: MAb directed either against IFN-γ or against IL-12 rescued mice from the shock reaction. Furthermore, Table 1 shows that $(p40)_2$ was able to protect mice in a dose-dependent manner. Intraperitoneal injection of $(p40)_2$ (100 μg/mouse) at the time of priming and 10 and 23 h later resulted in 95% survival of the animals. Application of less $(p40)_2$ (Table 1) and application of $(p40)_2$ fewer than three times (not shown) resulted in a decreased percentage of survival.

The TNFR IgG was shown to be protective in the LPS/D-GalN-induced shock syndrome (14) (see below). Table 1 demonstrates that TNFR IgG had no positive effect on survival in the experimental Shwartzman shock syndrome when given at the time of priming and during challenge.

IFN-γ has been shown to be necessary for the manifestation of lethality in the generalized Shwartzman reaction. Since $(p40)_2$ inhibits IL-12-induced IFN-γ production in vitro (11, 16), we examined the effect of $(p40)_2$ application on IFN-γ serum levels. For determination of IFN-γ, mice were sacrificed and heart blood was collected. Peak IFN-γ levels were reached 6 h after the LPS challenge injection. Figure 1 demonstrates that $(p40)_2$ decreased IFN-γ serum levels comparably to a MAb neutralizing IL-12. The average amount of IFN-γ serum level compared to the control group was reduced about fivefold in mice treated with anti-IL-12 reagents. These results clearly indicate that $(p40)_2$ is a potent IL-12 antagonist in vivo.

Homodimeric p40 prevents mortality in endotoxin-induced shock. To further confirm that $(p40)_2$ inhibited IL-12 activity in vivo, we investigated its influence in another LPS shock syndrome (LPS shock). In this model, groups of five C57BL/6 mice were injected i.p. with 350 μg of LPS (*S. abortus-equi*) per animal.

For pretreatment, mice received MAb neutralizing IL-12 activity or $(p40)_2$ 24, 12, and 1 h before LPS application. For determination of IFN-γ serum levels, blood was taken from the tail vein. As can be seen in Table 2, application of anti-IL-12 MAb 10F6 or $(p40)_2$ raised the survival rate to 90 and 93%, respectively, whereas only 12% of the mice in the PBS-treated group survived. In this model, TNFR IgG was found to have a partial positive effect on survival, because the proportion of survivors was raised to 70%. In addition, combined administration of TNFR IgG and IL-12 p40 homodimers was able to completely protect mice from LPS-induced shock (data not shown).

Anti-IL-12 MAb 10F6 as well as $(p40)_2$ were able to decrease dramatically peak serum IFN-γ levels, measured 8 h after LPS application (Fig. 2). IFN-γ serum levels were five- to

TABLE 2. Homodimeric IL-12 p40 prevents mortality in the endotoxin-induced shock syndrome

Treatment	No. of expt	No. of survivors/no. tested	% Survival
PBS	5	3/25	12
Anti-IL-12[a]	2	9/10	90[d]
$(p40)_2$[b]	3	14/15	93[d]
TNFR IgG[c]	2	7/10	70[d]

[a] 100 μg of rat anti-mIL-12 MAb 10F6 was administered i.p. 24, 12, and 1 h before LPS challenge.
[b] 100 μg of homodimeric p40 was administered i.p. 24, 12, and 1 h before LPS challenge.
[c] 20 μg of TNFR IgG was administered i.p. 1 h before LPS challenge.
[d] Significantly different ($P < 0.002$) from value for PBS-treated control group as calculated by Fisher's exact test.

TABLE 3. Homodimeric IL-12 p40 does not prevent mortality in the LPS/D-GalN-induced shock syndrome

Treatment	No. of expt	No. of survivors/no. tested	% Survival
PBS	4	0/20	0
Anti-IL-12[a]	2	0/10	0
$(p40)_2$[b]	2	2/10	20
TNFR IgG[c]	4	18/20	90[d]

[a] 100 μg of rat anti-mIL-12 MAb 10F6 was administered i.p. 24, 12, and 1 h before LPS/D-GalN challenge.
[b] 100 μg of homodimeric p40 was administered i.p. 24, 12, and 1 h before LPS/D-GalN challenge.
[c] 20 μg of TNFR IgG was administered i.p. 1 h before LPS/D-GalN challenge.
[d] Significantly different ($P < 0.0001$) from value for PBS-treated control group as calculated by Fisher's exact test.

sixfold lower in animals treated with IL-12 antagonists than in control mice. It should be mentioned that IFN-γ levels correlated well with survival or death of individual animals (data not shown). The potency of $(p40)_2$ as IL-12 antagonist in vivo was demonstrated again.

Homodimeric p40 does not prevent mortality in LPS/D-GalN-induced shock. The next investigated shock syndrome was induced by the application of LPS (0.1 μg/mouse) together with D-GalN (10 mg/mouse) to groups of five C57BL/6 mice.

For pretreatment, mice were injected i.p. with IL-12-neutralizing MAb or $(p40)_2$ 24, 12, and 1 h before, or with TNFR IgG 1 h before, application of LPS/D-GalN. TNFR IgG has been shown to be protective in this model (14). Table 3 shows that TNFR IgG raises the proportion of surviving animals to 90%, whereas no animal in the control group survived. Neither anti-IL-12 MAb 10F6 nor $(p40)_2$ had a protective effect in this model. In addition, MAbs neutralizing IFN-γ had no effect (not shown). IL-12 seems to play a minor role, if any, in the LPS/D-GalN-induced shock syndrome.

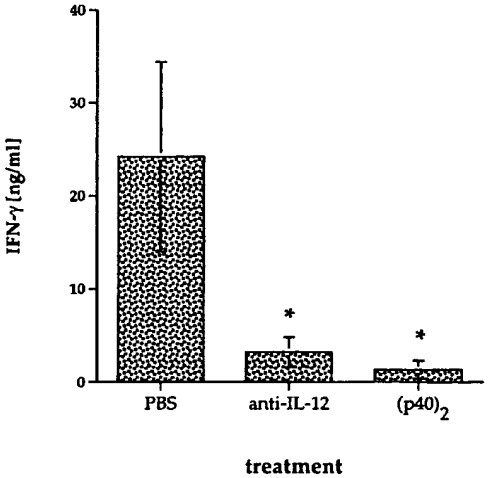

FIG. 2. Homodimeric p40 decreases IFN-γ serum levels in endotoxin-induced shock syndrome. Groups of 5 C57BL/6 mice were injected i.p. with 350 μg of LPS (*S. abortus-equi*). For pretreatment, mice received MAb neutralizing IL-12 (clone 10F6, 200 μg) or $(p40)_2$ (100 μg) 24, 12, and 1 h before LPS application. IFN-γ serum levels were determined 8 h after LPS injection. *, significantly different ($P < 0.001$) from value for PBS-treated control group as calculated by Student's *t* test.

DISCUSSION

Table 4 summarizes our findings. In experimental shock syndromes with IL-12 playing the central role, such as the Shwartzman reaction and endotoxin-induced shock, $(p40)_2$ is able to prevent mortality from mice, thus demonstrating its potency as an IL-12 antagonist in vivo. In both models, $(p40)_2$ strongly reduced IL-12-induced IFN-γ. In these models, TNFR IgG does not provide full protection even though a certain effect can be seen in endotoxin-induced shock. In contrast, TNFR IgG can rescue mice from LPS/D-GalN-induced shock, a model in which anti-IL-12 or anti-IFN-γ reagents have no effect.

Recently a new cytokine called IFN-γ-inducing factor (now called IL-18) was described (18). This factor was found to be critical in the induction of liver injury caused by endotoxin exposure of mice treated with *Propionibacterium acnes*. Treatment of mice with anti-IL-18 antibodies was able to prevent liver damage (18). In mice sensitized to the lethal effect of LPS by treatment with the avirulent bacille Calmette-Guérin (BCG) vaccine strain of *Mycobacterium bovis*, neutralizing anti-IL-12 antibodies were able to protect from shock-induced death (28). These studies suggest that IL-18 and/or IL-12 plays a critical role in development of septic shock. Potentially they act in synergy (17), and neutralizing one of both cytokines is sufficient for protection.

Our results obtained with MAb against IL-12 very closely parallel data published by Wysocka et al. for BCG-primed mice challenged with LPS (28). In this study, neutralizing anti-IL-12 antibodies were shown to inhibit IFN-γ production and mortality elicited by LPS. These results were confirmed by a recent report showing a fivefold reduction of serum IFN-γ levels in IL-12-deficient mice compared to wild-type mice upon challenge with LPS (15).

We have found significant reduction of serum IFN-γ levels after LPS challenge of mice treated with either anti-IL-12 MAb or homodimeric IL-12 p40. Preliminary data indicated that serum TNF-α was not reduced following treatment (data not shown). Similar results were published very recently by

TABLE 4. Prevention of shock by TNFR-IgG, homodimeric IL-12 p40, and anti-IFN-γ in different models

Shock model	Prevention of shock by:		
	TNFR-IgG	$(p40)_2$	Anti-IFN-γ
Shwartzman	No	Yes	Yes
LPS	Partial	Yes	Yes
LPS/D-GalN	Yes	No	No

Heinzel et al., who found significantly reduced serum levels of IFN-γ, but no change in the concentration of TNF-α and IL-12 heterodimer, in mice challenged with LPS and treated with recombinant homodimeric IL-12 p40 (12). These data suggest that homodimeric IL-12 p40 and anti-IL-12 MAb specifically block the interaction of IL-12 with its receptor, thereby antagonizing cellular activation, which results in selective inhibition of IFN-γ production. Interestingly, treatment with anti-IL-12 antibodies and homodimeric IL-12 p40, thereby targeting the ligand or the receptor, respectively, is similarly effective. Proinflammatory cytokines other than IFN-γ, such as TNF-α, appear to be regulated independently of IL-12. Moreover, our data show that selective inhibition of IL-12-induced IFN-γ production is sufficient to protect mice from the lethal effects of LPS.

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REFERENCES

1. **Alexander, H. R., G. M. Doherty, C. M. Buresh, D. J. Venzon, and J. A. Norton.** 1991. A recombinant human receptor antagonist to interleukin 1 improves survival after lethal endotoxemia in mice. J. Exp. Med. **173**:1029–1032.
2. **Basu, M., J. L. Pace, D. M. Pinson, M. P. Hayes, P. P. Trotta, and S. W. Russel.** 1988. Purification and partial characterization of a receptor protein for mouse interferon-γ. Proc. Natl. Acad. Sci. USA **85**:6282–6286.
3. **Billiau, A.** 1988. Gamma-interferon: the match that lights the fire? Immunol. Today **9**:37–40.
4. **Car, D. B., V. M. Eng, B. Schnyder, L. Ozmen, S. Huang, P. Gallay, D. Heumann, M. Aguet, and B. Ryffel.** 1994. Interferon γ receptor deficient mice are resistant to endotoxic shock. J. Exp. Med. **179**:1437–1444.
5. **Cerami, A., Y. Ikeda, N. Le Trang, P. J. Hotez, and B. Beutler.** 1985. Weight loss associated with an endotoxin-induced mediator from peritoneal macrophages: the role of cachectin (tumor necrosis factor). Immunol. Lett. **11**: 173–177.
6. **Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann.** 1987. Two types of mouse helper T cell clone. 3. Further differences in lymphokine synthesis between TH1 and TH2 clones revealed by RNA hybridisation, functionally monospecific bioassays and monoclonal antibodies. J. Exp. Med. **166**:1229–1244.
7. **Doherty, G. M., J. R. Lange, H. N. Langstein, H. R. Alexander, C. M. Buresh, and J. A. Norton.** 1992. Evidence for IFN-gamma as a mediator of the lethality of endotoxin and tumor necrosis alpha. J. Immunol. **149**:1666–1670.
8. **Galanos, C., M. A. Freudenberg, and W. Reutter.** 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76**:5939–5943.
9. **Gately, M. K., D. M. Carvajal, S. E. Connaughton, S. Gillessen, R. R. Warrier, K. D. Kolinsky, V. L. Wilkinson, C. M. Dwyer, G. F. Higgins, F. J. Podlaski, D. A. Faherty, P. C. Familletti, A. S. Stern, and D. H. Presky.** 1996. Interleukin-12 antagonist activity of mouse interleukin-12 p40 homodimer in vitro and in vivo. Ann. N. Y. Acad. Sci. **795**:1–12.
10. **Gately, M. K., A. G. Wolitzky, P. M. Quinn, and R. Chizzonite.** 1992. Regulation of human cytolytic lymphocyte responses by interleukin-12. Cell. Immunol. **143**:127–142.
11. **Gillessen, S., D. Carvajal, P. Ling, F. J. Podlaski, D. L. Stremlo, P. C. Familletti, U. Gubler, D. H. Presky, A. S. Stern, and M. K. Gately.** 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur. J. Immunol. **25**:200–206.
12. **Heinzel, F. P., A. M. Hujer, F. N. Ahmed, and R. M. Rerko.** 1997. In vivo production and function of IL-12 p40 homodimers. J. Immunol. **158**:4381–4388.
13. **Heinzel, F. P., R. M. Rerko, P. Ling, J. Hakimi, and D. Schoenhaut.** 1994. Interleukin-12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon. Infect. Immun. **62**:4244–4249.
14. **Lesslauer, W., H. Tabuchi, R. Gentz, M. Brockhaus, E. J. Schlaeger, G. Grau, P. F. Piguet, P. Pointaire, P. Vassalli, and H. Loetscher.** 1991. Recombinant soluble tumor necrosis factor receptor proteins protect mice from lipopolysaccharide-induced lethality. Eur. J. Immunol. **21**:2883–2886.
15. **Magram, J., S. E. Connaughton, R. R. Warrier, D. M. Carvajal, C.-Y. Wu, J. Ferrante, C. Stewart, U. Sarmiento, D. A. Faherty, and M. K. Gately.** 1996. IL-12 deficient mice are defective in IFN-γ production and type 1 cytokine responses. Immunity **4**:471–481.
16. **Mattner, F., S. Fischer, S. Guckes, S. Jin, H. Kaulen, E. Schmitt, E. Rüde, and T. Germann.** 1993. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. Eur. J. Immunol. **23**:2202–2208.
17. **Micallef, M. J., T. Ohtsuki, K. Kohno, F. Tanabe, S. Ushio, M. Namba, T. Tanimoto, K. Torigoe, M. Fujii, M. Ikeda, S. Fukuda, and M. Kurimoto.** 1996. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. Eur. J. Immunol. **26**:1647–1651.
18. **Okamura, H., H. Tsutsui, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, K. Akita, M. Namba, F. Tanabe, K. Konishi, S. Fukuda, and M. Kurimoto.** 1995. Cloning of a new cytokine that induces IFN-γ production by T cells. Nature **378**:88–91.
19. **Ozmen, L., M. Pericin, J. Hakimi, R. A. Chizzonite, M. Wysocka, G. Trinchieri, M. Gately, and G. Garotta.** 1994. Interleukin 12, interferon γ, and tumor necrosis factor α are the key cytokines of the generalized Shwartzman reaction. J. Exp. Med. **180**:907–915.
20. **Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. S. Ohashi, M. Kronke, and T. W. Mak.** 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell **73**:457–467.
21. **Prat, M., G. Gribaudo, P. Comoglio, G. Cavallo, and S. Landolfo.** 1984. Monoclonal antibodies against murine gamma interferon. Proc. Natl. Acad. Sci. USA **81**:4515–4519.
22. **Rothe, J., W. Lesslauer, H. Loetscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann.** 1993. Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature **364**:798–802.
23. **Starnes, H. F., M. K. Pearce, A. Tewari, J. H. Yim, J. Zou, and J. S. Abrams.** 1990. Anti-IL-6 monoclonal antibodies protect against lethal Escherichia coli infection and lethal tumor necrosis factor-alpha challenge in mice. J. Immunol. **145**:4185–4191.
24. **Thomas, L., and R. A. Good.** 1952. Studies on the generalized Shwartzman reaction. I. General observations concerning the phenomenon. J. Exp. Med. **96**:605–611.
25. **Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science **234**:470–474.
26. **Ulich, T. R., K. Guo, B. Irwin, D. G. Remick, and G. N. Davatelis.** 1990. Endotoxin-induced cytokine gene expression in vivo. II. Regulation of tumor necrosis factor and interleukin-1 alpha/beta expression and suppression. Am. J. Pathol. **137**:1173–1185.
27. **Wilkinson, V. L., R. R. Warrier, T. P. Truitt, P. Nunes, M. K. Gately, and D. H. Presky.** 1996. Characterization of anti-mouse IL-12 monoclonal antibodies and measurement of mouse IL-12 by ELISA. J. Immunol. Methods **189**:15–24.
28. **Wysocka, M., M. Kubin, L. Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri.** 1995. Interleukin-12 is required for interferon-γ production and lethality in lipopolysaccharide-induced shock in mice. Eur. J. Immunol. **25**: 672–676.

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IL-12, Independently of IFN-γ, Plays a Crucial Role in the Pathogenesis of a Murine Psoriasis-Like Skin Disorder

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The onset of acute psoriasis and the exacerbation of chronic psoriasis are often associated with a history of bacterial infection. We demonstrate that while only few *scid/scid* mice develop disease when $CD4^+CD45Rb^{high}$ T cells are transferred alone, coadministration of LPS plus IL-12 or staphylococcal enterotoxin B into *scid/scid* mice 1 day after $CD4^+CD45Rb^{high}$ T cell transfer greatly enhances disease penetrance and severity. Most importantly, the skin lesions induced by this method exhibit many of the histologic hallmarks observed in human psoriasis. Skin infiltrating $CD4^+$ T cells were predominantly memory/effector cells ($CD45Rb^{low}$) and exhibited a highly polarized Th1 phenotype. To test whether the development of pathogenic T cells was dependent on their production of IFN-γ, we transferred IFN-$\gamma^{-/-}$ $CD4^+CD45Rb^{high}$ T cells into *scid/scid* or into T, B and NK cell-deficient *scid/beige* mice. Surprisingly, the incidence of psoriasis was similar to *scid/scid* animals that received IFN-$\gamma^{+/+}$ T cells, although acanthosis of the skin was attenuated. In contrast, the development of psoriasis was abolished if anti-IL-12 mAb was administered on day 7 and 35 after T cell transfer. Skin-derived IFN-$\gamma^{-/-}$ inflammatory cells, but not cells from anti-IL-12-treated animals, secreted substantial amounts of TNF-α, suggesting that the inflammatory effect of IFN-$\gamma^{-/-}$ T cells may be partly exerted by TNF-α and that the therapeutic effect of anti-IL-12 may depend on its ability to down-regulate both TNF-α and IFN-γ. Overall, these results suggest that IL-12, independently of IFN-γ, is able to induce pathogenic, inflammatory T cells that are able to induce psoriasiform lesions in mice. *The Journal of Immunology,* **1999, 162: 7480–7491.**

Psoriasis is a chronic inflammatory skin disease that is associated with hyperplastic epidermal keratinocytes and infiltrating mononuclear cells, including $CD4^+$ memory T cells, neutrophils, and macrophages (reviewed in Refs. 1–3). Because of this highly mixed inflammatory picture and the resulting complex interrelationships between these different cells, it has been very difficult to dissect the mechanisms that underlie the induction and progression of the disease. Indeed, whether genetically determined alterations in keratinocyte proliferation or immunoregulatory defects are the primary cause of psoriasis is currently unresolved. Some investigators believe that environmental factors, such as microbial infection and trauma, can be an initiating event in the pathogenesis of the disease (4–7). This hypothesis also implies that although dormant autoreactive T cells may pre-exist in susceptible individuals, an environmental stimulus is necessary to trigger disease induction. Others believe that the immune system plays only a minor modulatory role in the disease process and that hyperproliferation of keratinocytes is in fact the initiating event in a genetically susceptible host.

Research into the pathogenesis of psoriasis has long been hindered by the lack of suitable animal models. Although several rodent models of skin inflammation have been recently introduced, in none of these models have specific T cell abnormalities been demonstrated as a primary cause for the induction of disease (8–14). Most recently, Schon et al. (15) presented evidence that a particular splenic T cell subset ($CD4^+CD45Rb^{high}$), the same T cell subset that induces colitis in *scid/scid* mice, is able to induce psoriasis-like lesions when transferred into a minor haplotype mismatched *scid/scid* mice. Other investigators have demonstrated that when pre-psoriatic skin, but not skin from healthy donors, is engrafted onto *scid/scid* mice, the transplanted skin develops into psoriasiform lesions after autologous blood-derived immunocytes are activated by staphylococcal enterotoxin B (SEB)[3] and IL-2 and injected into the dermis (16, 17). In addition, patients that received fragments of diphtheria toxin linked to human IL-2 (DAB389IL-2), which selectively targets activated T cells but not keratinocytes, showed significant clinical improvement, indicating that T cells and not keratinocytes are the primary pathogenic component in the disease (18). Although these observations provide enough first evidence to support the concept that psoriasis-like conditions can indeed result from unregulated T cell responses, they provide very little evidence on the specific mechanism and the cytokines that are involved in the induction of psoriasiform lesions.

Bacteria and their products have been implicated as an initiating event in various T cell mediated autoimmune conditions in humans, including rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) (6, 19). For example, in murine models of RA and IBD, mice do not develop inflammatory lesions under germfree conditions (20–25). Indeed, either normal luminal bacteria (26) or infection with a single microbial pathogen have been shown to significantly increase the expression of disease (27, 28). LPS and SEB are important bacterial-derived immunomodulators, since they are not only able to activate immune-competent cells but are also able to increase the expression of cell adhesion molecules on vascular endothelial cells and T cells and thereby promote the entry of inflammatory cells into tissues (29–35). IL-12 produced very early during infection in vivo has important proinflammatory functions. It plays a key role in the differentiation of naive T cells

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[3] Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; IFN-$\gamma^{-/-}$, IFN-γ-deficient mice; SIL, skin infiltrating lymphocytes.

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into IFN-γ-producing Th1 cells (36) and thus seems to be important in the induction of many T cell-mediated autoimmune diseases (21, 37–42).

The role of IFN-γ in autoimmunity has been more controversial. Although IFN-γ production seems to be a hallmark of inflammatory T cells involved in numerous autoimmune conditions, it is less clear what function IFN-γ actually plays in the disease process. Studies in animal models of inflammation and autoimmunity and in humans revealed that IFN-γ can have opposing immunosuppressive or immunostimulatory effects depending on the disease and the time of application (reviewed in Ref. 43). In psoriasis, IFN-γ is thought to play an important primary role in the disease pathogenesis, since T cells isolated from psoriatic lesions of patients secrete high amounts of IFN-γ (44, 45) and T cell clones obtained from psoriatic skin directly promote keratinocyte proliferation through an IFN-γ-dependent pathway (46).

In this study, we further dissect the pathogenic mechanism of psoriatic lesions by asking what role IL-12 and IFN-γ play in the induction of pathogenic T cells in psoriasis. In particular, we ask whether IL-12, which in itself is an important up-regulator of IFN-γ production, is able to induce chronic psoriasiform skin inflammation in the absence of IFN-γ. We demonstrate that the injection of bacterial products and IL-12 significantly enhances penetrance and severity of psoriasiform lesions in this newly described murine model of psoriasis. Furthermore, we demonstrate that the pathogenesis of disease is driven by an IL-12-dependent, but IFN-γ-independent mechanism, suggesting a novel strategy for therapeutic intervention in patients with psoriasis.

Materials and Methods

Mice

Female BALB/c mice and BALB/c-IFN-$\gamma^{-/-}$ mice (donor mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). C.B-17/Icr *scid/scid* mice and C.B-17 *scid/beige* double mutant mice (recipient mice) were purchased from Taconic Farms (Germantown, NY). All mice were housed in a specific pathogen-free environment at the Protein Design Labs animal facility and were used between 4 and 12 wk of age. Sentinel mice were used to screen for the following pathogens: Mouse hepatitis virus (MHV), Sendai virus, Pneumonia virus of mice (PVM), Reovirus serotype 3 (REO3), Theiler's murine encephalomyelitis virus (TMEV), *Myco-plasma. pulmonis*, and parvovirus. Random screens of mice for pinworms were also conducted. None of the pathogens listed above were detected at any time. Mice were housed 2–5/microisolator. All *scid/scid* or *scid/beige* mice were handled with gloves under a class II hood, fed sterile food and water ad libitum, and maintained inside a laminar flow tent (Bioclean, Maywood, NJ) in sterile microisolators that were changed weekly. Donor mice were housed in conventional cages that were changed weekly.

Cell purification, and injection into scid/scid *mice*

Spleens were collected from 6- to 12-wk-old donor mice (BALB/c or IFN-$\gamma^{-/-}$-BALB/c) and splenocytes were isolated by mechanical homogenization of whole spleens. $CD4^+$ T cells were selected by positive selection. In brief, a cell suspension of pooled splenocytes from four to five donor mice was incubated with anti-CD4 (L3T4) Ab coated magnetic beads (Dynabeads, catalog no. 114.05, Dynal, Lake Success, NY) for 20–30 min at 4°C and separated by magnetic cell sorting with a Dynal Magnetic Particle Concentrator (MPC). Cells were removed from the cell-bead complex with Dynal DETACHaBEAD, and isolated from beads using a Dynal MPC. The resulting $CD4^+$ enriched population was >90% pure. The cell suspension (10×10^6 cells/ml) was then incubated with Fc block (anti-CD32, 01241A; PharMingen, San Diego, CA) (10 μg/ml) and labeled with anti-CD4-FITC (9004D; PharMingen) and anti-CD45RB-PE (01145A; PharMingen) (both at 10 μg/ml) for 30 min at 4°C, washed, and sorted using a FACStar (Becton Dickinson, San Jose, CA) cell sorter. Double positive cells ($CD4^+CD45Rb^+$) were collected, selecting the cells that expressed high levels of CD45Rb (brightest 45%). The collected cell population was >90% pure and viable. Cells were then washed in cold PBS (D8662; Sigma, St. Louis, MO) and resuspended in PBS at 1.5×10^6 cells/ml. C.B-17/Icr *scid/scid* mice, aged 4–6 wk, were injected i.v. with 3×10^5 cells each, 200 μl total volume into the tail vein.

Induction and treatment of psoriasiform lesions in scid *mice*

To study the effect of microbial products and IL-12, recipient mice were treated as follows. A control group received $CD4^+CD45Rb^{high}$ sorted cells with no additional treatment. A second group was given 20 μg LPS from *Salmonella enteritidis* (Sigma; L-2012) s.c. or i.p. per mouse on day 1 after cell transfer. A third group received 10 ng IL-12 (PharMingen) alone per mouse delivered s.c. or i.p. on days 1 and 3. The final group was injected s.c. or i.p. with a combination of LPS and IL-12. Dosage studies were conducted using 2, 10, and 100 ng doses of IL-12 in conjunction with 20 μg LPS. The LPS and IL-12 injection was given on day 1 following T cell transfer and an additional dose of IL-12 was administered on day 3. In additional studies, LPS (20 μg) and IL-12 (10 ng) were administered once weekly for 3 wk. Some experimental groups received 10 μg SEB protein from *Staphylococcus aureus* (Sigma S4881) i.p. per mouse once on day 1 following T cell transfer.

To study the role of IFN-γ, T cells from BALB/c-IFN-$\gamma^{-/-}$ mice (The Jackson Laboratory) T cells were isolated by the same methods described above. Recipient *scid/scid* mice were also coinjected with 20 μg LPS and 10 ng IL-12 on day 1 and 10 ng of IL-12 on day 3. In addition, *scid/beige* mice (Taconic Farms), that are T, B, and NK cell deficient, were used as recipient mice for IFN-$\gamma^{-/-}$ T cell transfer in some experiments. For interventional studies, 0.5 mg anti-IL-12 (clone C17.8; PharMingen) was given i.p. to mice on day 7 and 35. Control mice received either PBS or rat IgG (Sigma) on the same day.

Clinical evaluation

Mice were evaluated by three different investigators at weekly intervals commencing on week 4 and ending on week 10. To record disease progression semiquantitative clinical scores from 0 to 4 were given based on physical appearance and ear thickness: 0 = no skin or ear symptoms; 1 = mild, moderate erythema on ears or eyelids with mild thickening of the ear (<2% of the body surface); 2 = moderate to severe erythema on one location (mostly ear and face) (2–10% of the body surface), mild scaling; 3 = severe erythema at two or more sites (ear, face, trunk) (>10% of the body surface), severe scaling; 4 = very severe, extensive erythema throughout the body (>20% of the body surface) with severe scaling. Specific observations were noted based on fur condition, ear manifestations, eyelid appearance, and presence of inflammation on limbs and tail. Ear thickness was determined using a modified spring micrometer (Oditest; Dyer, Lancaster, PA). Measurements were taken from the same part of the ear for all data time points from both the right and left ear. The micrometer was allowed to settle while on the ear to prevent tissue edema from affecting final measurement.

Histopathologic analysis

Necropsies were performed on mice at week 10–12 after cell transfer. Tissue samples from ear, eyelid, and tail were collected and fixed in paraformaldehyde solution and submitted to Comparative Bioscience (Sunnyvale, CA) for section preparation and analysis. To record disease severity, semiquantitive histological scores from 0 to 4 were given based on the severity of inflammation. Initial histological evaluation was performed in a blinded fashion by an independent outside pathologist. In later studies evaluation was blindly conducted by three different investigators. Mice which had ear thickness of 25 μm or less with no additional clinical signs were automatically given a histology score of zero without section analysis: 0 = no signs of inflammation; 1 = very low focal areas of infiltration, mild acanthosis; 2 = low level of mononuclear cell infiltration, mild thickening of epidermis, mild to moderate acanthosis; 3 = high level of mononuclear cell infiltration, high vascular density, thickening of the epidermis (acanthosis, rete pegs and hyperplasia of epidermis and keratinocytes, microabscesses, thinning of the granular cell layer); 4 = very extensive infiltration in epidermis and dermis, very high vascular density, extreme thickening of epidermis, pustule formation and destruction of granular cell layers.

Tissue samples were collected and embedded in Tissue Tek OCT (Miles, Elkhurt, IN) compound and frozen with dry ice for cryostat-cut sections. Tissue sections (5 μm) were fixed in 100% acetone and stained with PE-conjugated IL-12 mAb (p40/70) (PharMingen, clone C17.8). Tissues were evaluated as positive or negative based on visual fluorescent microscopy detection.

Skin infiltrating lymphocyte cell isolation

Skin infiltrating lymphocytes were isolated via enzyme digestion. In short, skin, ears, and eyelids were minced with sterile scissors, and the pieces were washed with HBSS over a 100-mm nylon cell strainer (Falcon, Becton Dickinson, Franklin Lake, NJ) to remove surface debris. Infiltrating cells were liberated by incubating the cut pieces in 25 ml of warm (37°C)

Table I. *In vivo administration of IL-12 in combination with LPS after CD4$^+$CD45Rb^{high} T cell transfer leads to a significant increase in disease expression*

Post Cell Transfer Treatment[a]	Disease Incidence[b]	Average Histology Score[c]	Average Time of Disease Onset (wk)[d]	Severe Disease Incidence[e]
PBS	10/26 (38%)	1.1 ± 1.4	7.3 ± 2.0	7/26 (27%)
IL-12 medium	2/3 (67%)	0.75 ± 1.0	10.5 ± 2.1	0/3 (0%)
LPS	2/4 (50%)	0.6 ± 0.7	8.5 ± 2.1	0/4 (0%)
LPS + IL-12 low	6/11 (55%)	1.9 ± 1.0	6.7 ± 0.8	4/11 (36%)
LPS + IL-12 medium	24/33 (73%)**	2.25 ± 1.1*	6.2 ± 1.7	14/33 (42%)*
LPS + IL-12 high	0/4 (0%)	0.0	ND	0/4 (0%)
LPS + IL-12 medium[f]	8/10 (80%)**	2.5 ± 1.0*	4.7 ± 1.0	5/10 (50%)**
LPS + IL-12 medium[g]	0/8 (0%)	ND	ND	0/8 (0%)
SEB	3/4 (75%)	ND	8.0 ± 2.0	2/4 (50%)

[a] After CD4$^+$CD45Rb^{high} cells isolated from BALB/c spleens were transferred to *scid/scid* mice, the recipient mice were given the following treatments. The PBS group received sorted cells with no additional treatment. The IL-12 group received 10 ng IL-12 alone per mouse delivered i.p. on days 1 and 3 in addition to cells on day 0. The LPS group was given 20 μg LPS from *Salmonella enteritidis* (Sigma; L-2012) per mouse on day 1 after cell transfer. Dosage studies were conducted using the low (2 ng), medium (10 ng), or high (100 ng) doses of IL-12 in conjunction with 20 μg LPS. The LPS and IL-12 injections were given on day 1, and an additional dose of IL-12 was administered on day 3. In a different series of experiments LPS (20 μg) and IL-12 (10 ng) were injected once a week for 3 wk. Other mice received 10 μg SEB protein from *Staphylococcus aureus* (Sigma catalog S4881) per mouse once on day 1.

[b] Disease incidence is reported as number of mice with disease over 12 wk of time: criteria being ear thickness ≥ 26 μm or clinical score of ≥ 1. Ear thickness of normal *scid/scid* mice: 21 ± 1 μm (n = 10). The statistical significance between different treatment groups was analyzed using the χ^2 test. Values for p vs PBS control: **, $p < 0.001$.

[c] Histological scores of diseased mice from 1 to 4 were given based on the severity of inflammation of ear, skin, or eyelid. Mice that had ear thickness of 25 μm or less were categorized as undiseased (average ear thickness of undiseased mice, 22 ± 1 μm) and automatically excluded from section analysis: 0 = no signs of inflammation; 1 = very low level; 2 = low level of mononuclear cell infiltration, mild thickening of epidermis; 3 = high level of mononuclear cell infiltration, high vascular density, thickening of the epidermis (acanthosis, rete pegs, and hyperplasia of epidermis and keratinocytes); 4 = very extensive mononuclear cell infiltration in epidermis and dermis, very high vascular density, extreme thickening of epidermis, pustule formation, and loss of granular cell layers. Values for p vs PBS control: *, $p < 0.01$. Statistical analysis was performed using the two-tailed Student's t test.

[d] Average time of disease onset is reported as the number of weeks after cell transfer before clinical signs of disease were observed. Data represent diseased mice only.

[e] Severe disease induction calculated as the number of animals that received an average histology score of ≥2.5 and/or a clinical score of ≥3. The statistical significance between different treatment groups was analyzed using the χ^2 test. Values for p vs PBS control: *, $p < 0.01$, and **, $p < 0.001$.

[f] LPS (20 μg) and IL-12 (10 ng) was given once a week for 3 wk.

[g] A total of 1 × 10^6 unsorted CD4$^+$ T cells (CD45Rb^{high} and CD45Rb^{low}) were transferred into *scid/scid* mice. LPS (20 μg) and IL-12 (10 ng) was given once a week for 3 wk.

HBSS media without Ca^{2+}/Mg^{2+} (10-543F; BioWhittaker, Walkersville, MD) supplemented with 25 mM HEPES buffer (17-737E; BioWhittaker) and 10% *FBS* (HyClone, Logan, UT SH30071.03) for 20 min at 37°C. The remaining pieces were washed over nylon mesh, resuspended in RPMI 1640 medium (12-702F; BioWhittaker) augmented with 25 mM HEPES buffer, 10% *FBS*, 400 U/ml DNase (104159; Boehringer Mannheim, Indianapolis, IN), 400 U/ml collagenase (1088874; Boehringer Mannheim), and incubated 90 min at 37°C on a rocker. The resulting cell suspension was filtered sequentially through a 100-μm and 40-μm nylon mesh filter and then washed twice in RPMI 1640 medium supplemented with 25 mM HEPES and 10% *FBS*.

In vitro stimulation of skin infiltrating lymphocytes (SIL) and detection of cytokines

SIL were resuspended at 10^6/ml in complete RPMI 1640 medium supplemented with 10% FBS (HyClone), 5 × 10^{-5} M 2-ME (Sigma), 2 mM glutamine (Life Technologies, Gaithersburg, MD), 10 U/ml penicillin, 100 μg streptomycin (Life Technologies), and 15 mM HEPES. CD4$^+$ sorted T cells were resuspended at 2.5 × 10^5/ml. A total of 200 μg/well of this suspension was then placed in a 96-well tissue culture plate to (3072 Falcon) and incubated for 48 h with anti-CD3 (clone 145-2C11; Protein Design Labs) and anti-CD28 (PharMingen), each at 1 μg/ml. Supernatants from three different culture wells were collected and tested by ELISA for IFN-γ, TNF-α, and IL-4. The ELISA procedure involved coating a 96-well flat-bottom Immulon 4 plate (011-010-3850; Dynatech, Chantilly, VA) overnight at 4°C with 50 μl of a 2 μg/ml solution of anti-IFN-γ, anti-TNF-α, or anti-IL-4 Ab (all from PharMingen) in carbonate buffer. Plates were then washed with PBS/Tween (0.05% Tween-20 in PBS) and blocked with 200 μl sterile solution of PBS with 3% BSA to (A7030 Sigma Bovine Albumin) for 1 h at 37°C. In between all of the following steps, plates were washed with PBS/Tween. IFN-γ, IL-4, and TNF-α standards as well as sample supernatants were then added to wells and incubated for 2 h at 37°C. Biotin-conjugated secondary Abs for anti-IFN-γ, anti-TNF-α, and anti-IL-4 (all Abs from PharMingen) were then added to the respective plates at 2 μg/ml in 3% BSA/PBS solution and incubated for 1 h at 37°C. HRP-labeled streptavidin (016-030-084; Jackson ImmunoResearch, West Grove, PA) was then added at a concentration of 1 μg/ml. *O*-Phenylenediamine to (4664 Sigma) was then used as substrate buffer per manufacturer's protocol. Assay was then read on a Molecular Devices (Sunnyvale, CA) plate reader and data were analyzed using SOFTmax software.

Statistical analysis

Descriptive statistics and testing for significance of differences between treatment groups were assessed either by the two-tailed Student's t test or by using the χ^2 test using the Microsoft Excel (Redmond, WA) statistical program.

Results

Treatment of scid/scid *mice restored with CD4$^+$CD45Rb^{high} T cells with LPS plus low and medium doses of IL-12 results in increased incidence and severity of psoriasis: high doses of IL-12 prevent disease induction*

Previous studies have demonstrated that *scid/scid* mice reconstituted with minor haplotype mismatched CD4$^+$CD45Rb^{high} BALB/c T cells sometimes develop chronic skin inflammation that resembles human psoriasis (15). In initial experiments, we found that when BALB/c CD4$^+$CD45Rb^{high} T cells alone were transferred to C.B-17 *scid/scid* mice, only a few animals exhibited psoriasiform lesions and the expression of disease was rather mild. This finding was consistent with previous observations made by Schon et al. (15). Because bacterial mitogens or bacterial superantigens have been shown to be potent modulators of cell-mediated immune responses, and IL-12 has been demonstrated to play an important role in the induction of various autoimmune conditions (reviewed in Refs. 36, 42, and 47), we tested initially whether the coadministration of such agents would have an effect on the induction of psoriasis in the *scid/scid* transfer model. As shown in Table I, when C.B-17 *scid/scid* mice were reconstituted with

BALB/c $CD4^+CD45Rb^{high}$ T cells alone, only 38% of the mice developed psoriasiform skin lesions and only 27% of the mice developed severe forms of disease. When LPS was coadministered alone, we observed a slight, but insignificant, increase in disease incidence (50%), and moreover the severity of the lesions remained similar to lesions in mice that had received cells alone. Similarly, coadministration of a medium dose (10 ng/mouse) of IL-12 alone on day 1 and 3 following T cell transfer led to an apparent increase in disease incidence (67%) without affecting disease severity.

In contrast, recipient mice that received either 1 or 10 ng IL-12 on day 1 and day 3 along with 20 μg of LPS on day 1 following T cell transfer showed an increase in disease severity and incidence. In particular, the administration of a medium doses of IL-12 (10 ng/mouse) along with LPS showed a 73% incidence of disease with an average histology score of 2.25 $\pm$ 1.1, which was significantly higher than when PBS was coadministered ($p < 0.008$). In addition, the percentage of animals with severe disease in the IL-12 medium dose group was also higher (42%) when compared with the IL-12 low dose group (36%) and significantly higher when compared with the PBS control (27%). Interestingly, coadministration of LPS (20 μg/mouse) and a high dose of IL-12 (100 ng/mouse) completely inhibited disease development (incidence 0%). When LPS and medium doses of IL-12 (10 ng) were coadministered once a week for 3 wk the incidence of disease was 80% (8/10) with an average clinical score of 2.5 $\pm$ 1 (Table I). This particular induction protocol was also associated with an accelerated onset of disease as the animals in this group came down with disease as soon as 4 wk after T cell transfer (average time of onset, 4.7 $\pm$ 1.0 wk). In contrast, animals that had received only one dose of LPS and IL-12 (10 ng) developed disease at an average of 6.2 $\pm$ 1.7 wk after T cell transfer, and animals that received T cells only developed signs of disease at an average of 7.3 $\pm$ 2.0 wk after T cell transfer.

Animals that had received unsorted $CD4^+$ T cells (1×10^6/mouse) never came down with disease even if they were treated with three administrations of LPS (20 μg) and IL-12 (10 ng) (Table I), indicating that LPS and IL-12 administration can only act on naive T cells and the regulatory effects of $CD45Rb^{low}$ cells cannot be overcome by the administration of microbial factors and IL-12. To ensure the presence of sufficient numbers of effector T cells in the unsorted cell population, we transferred up to 5×10^6 unsorted $CD4^+$ T cells plus LPS and IL-12 three times to naive *scid/scid* mice in a different set of experiments; however, this approach also failed to induce disease lesions (data not shown). Of note, mice that received no T cells or T cells alone were housed together with mice that received T cells plus LPS and IL-12, to ensure that other exogenous factors did not play a role in the induction of disease.

In a different set of experiments, we tested whether other microbial products such as SEB exert an influence on disease expression as well. As shown in Table I, SEB was also able to induce disease at a higher incidence and severity (average clinical score, 1.5 $\pm$ 0.9) than cells alone, thus demonstrating that the ability of bacterial constituents to modulate the expression of psoriasiform lesions is not unique to LPS.

In separate cell transfer studies, we found that *scid/scid* mice that received inflammatory cells isolated from the skin lesions of diseased mice (4×10^6 cells were administered/mouse) did not develop psoriasis unless LPS and IL-12 was coadministered (data not shown), indicating that the transfer of inflammatory psoriatic T cells alone is not sufficient to induce a chronic inflammatory response in the skin.

Psoriatic skin lesions of scid/scid *mice treated with LPS and IL-12 closely resemble human pathology*

Animals that received $CD4^+CD45Rb^{high}$ cells in conjunction with LPS and IL-12 developed disease symptoms as soon as 4–8 wk after cell transfer (see Table I). Mice without clinical signs of disease at week 10 post T cell transfer remained disease free for an additional 4–6 wk of observation. Thus, mice were monitored beginning on week 4, and necropsies were performed on subject animals between weeks 10 and 12. Clinical signs of disease consistently included increased erythema of the ear and thickened skin on ears and eyelids. Some animals also showed signs of significant skin inflammation on the tail. In more severe cases, skin inflammation was observed throughout the body with increased scaling and hair loss (clinical score 4) (Fig. 1*J*). Ear thickness typically varied from a baseline of 21 $\pm$ 1.1 μm in undiseased animals to a pathological range of 26–50 μm. Skin that became severely affected consistently became scaly, ulcerated, and typically showed plaque-type elevation (see also Fig. 1*J*). Skin inflammation in psoriatic mice ranged from mild, around the base of the ears and around the eyelids (clinical score 1–2), to severe hair loss that extended to over 75% of their body (clinical score 3–4) (see *Materials and Methods*). Since ear thickness correlated very well with the severity of disease and clinical scores, we used the measurement of ear thickness as an indicator for overall skin inflammation in most experiments.

Other psoriasis-like models in mice have been criticized for not possessing the histological characteristics found in human forms of disease. Differences in the mouse skin structures were deemed responsible for these discrepancies. An absence of elongation of rete pegs (i.e., down-growths of epidermis into dermis) in mice, a major hallmark in human disease, was attributed to the relatively flat dermoepidermal junction in mice (8, 48). Histological analysis of the lesions induced by our protocol was performed by taking biopsies of skin samples from several areas of diseased mice and examining 3-μm sections stained with hematoxylin and eosin. Samples taken from the ear, eyelid, and tail of diseased mice that had received T cells plus LPS (20 μg/mouse) and IL-12 (10 ng/mouse) were prepared for histologic evaluation and microscopically examined by an independent pathologist. Most lesions were found to have typical signs of hyperkeratosis, including orthokeratosis (i.e., layers of anucleated cornified cells) and parakeratosis (i.e., layers of nucleated cornified cells) with some ulcer or erosion and pustule formation (Fig. 1, *D* and *H*). Also noted was a thickening of the epidermis (acanthosis) with proliferation of the keratinocytes and moderately deep rete pegs in the subcutis (Fig. 1, *D* and *E*); the inflammatory cell infiltration consisted of primarily mononuclear cells composed of lymphocytes with fewer monocytes, macrophages and plasma cells. Variable numbers of neutrophils with a few eosinophils were also seen. Capillaries and other vessels were numerous, contained large numbers of marginated neutrophils and were typically surrounded by lymphocytes (Fig. 1, *D–H*). The combination of these characteristics indicate that the psoriasiform lesions in this model are very comparable to those found in humans. Immunohistochemistry staining of the skin and FACS analysis of infiltrating lymphocytes revealed that beside numerous $CD4^+$ T cells, no endogenous $CD8^+$ T cells were present in the skin lesions (data not shown). Fig. 1, *A–C*, is representative of the histology found in skin from normal *scid/scid* mice that received no T cells. Sections from these mice were taken at the same age as diseased mice. The epidermis is 1–2 cells thick and the dermis contains almost no lymphocytes. In addition, the density of vessels is sparse. The junction of the dermis to the epidermis is straight and contains no abscesses.

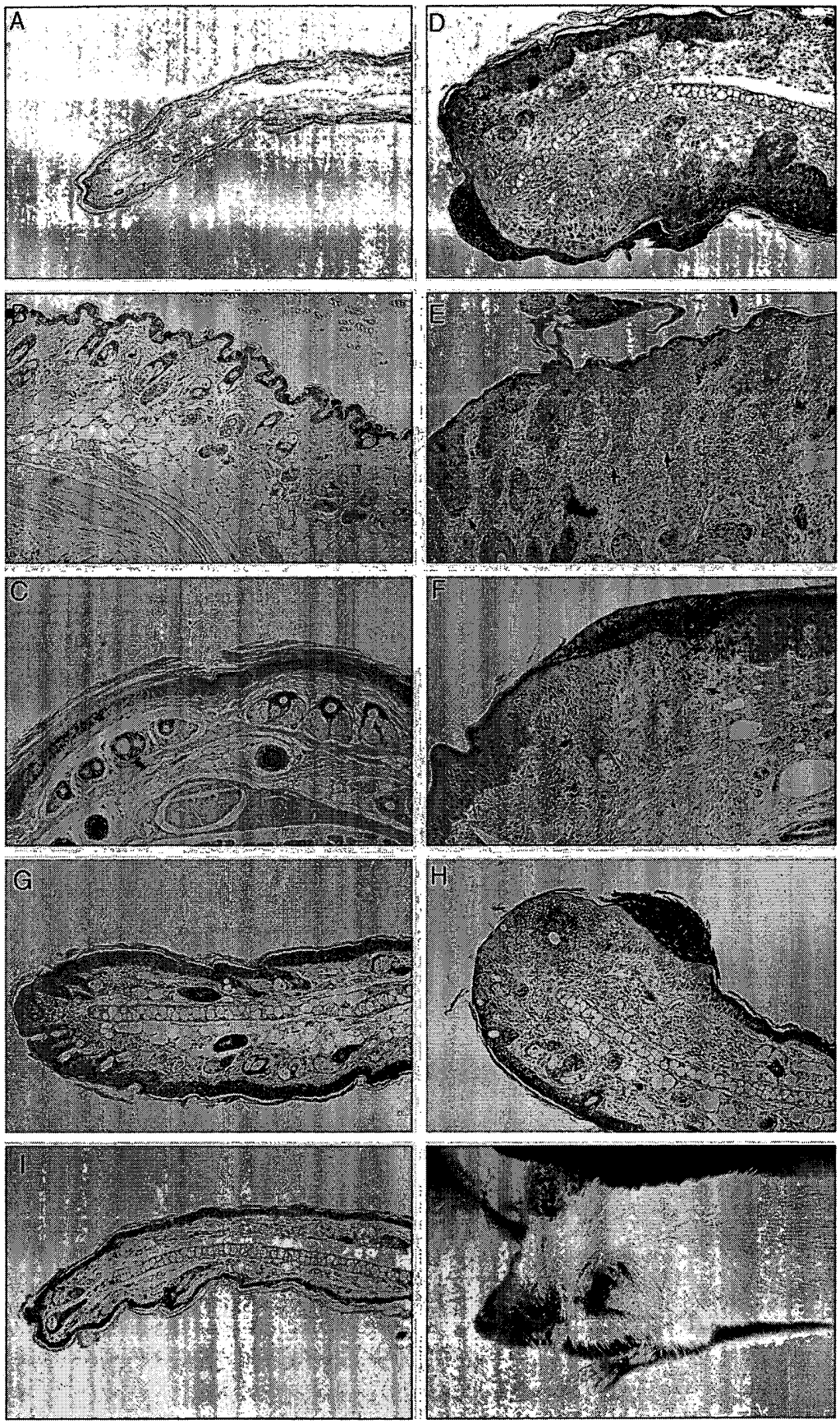

FIGURE 1. Histological analysis of psoriasiform lesions in CD45Rb^{high} T cell reconstituted *scid/scid* mice and treated with LPS and IL-12. All cross-sections were taken at ×100 magnification. Tissue from normal *scid/scid* mice. *A–C*, Ear, eyelid, and tail; magnification ×100. Tissue from *scid/scid* mice that received CD4^{+}CD45Rb^{high} T cells in combination with LPS (20 μg) and IL-12 (10 ng). *D*, Ear; note the presence of the major hallmarks of psoriasis: acanthosis (i.e., thickening of the epidermis) and hyperkeratosis (i.e., thickening of the cornified layers) with some ulcer or erosion formation and

Table II. *$CD4^+$ T cells isolated from psoriatic lesions of* scid/scid *mice produce high levels of interferon-γ and low levels of IL-4*[a]

T Cell Source	IFN-γ (pg)	IL-4 (pg)	TNF-α (pg)
Skin from *scid/scid*-diseased[b]	22,987 ± 648	468 ± 79	4,654 ± 946
Skin from *scid/scid*-undiseased[c]	≤20	≤15	≤35
$CD4^+CD45RB^{high}$ T cells from BALB/c spleens[d]	≤20	≤15	≤35

[a] IFN-γ, IL-4, and TNF-α production was measured as described in *Materials and Methods*. Data represents the mean ± SD.
[b] Cells (2.0×10^5) from psoriasiform lesions of 5–10 recipient *scid/scid* mice that had developed disease following reconstitution with $CD4^+CD45Rb^{high}$ cells from normal BALB/c mice along with LPS and IL-12 were cultured for 48 h. Data are one value representative of three experiments showing similar values.
[c] Cells (2.0×10^5) from the skin of 8–10 recipient *scid/scid* mice that did not show disease symptoms were cultured for 48 h. Data are from one experiment representative of two independent experiments showing similar values.
[d] $CD4^+CD45RB^{high}$ cells (2.0×10^5) sorted by flow cytometry from spleen cells isolated from BALB/c mice were cultured for 48 h. Cell purity was ≥90%.

Of note, as one might expect from this model, various degrees of inflammation were observed in the colon of these animals as well. Interestingly, the administration of LPS and IL-12 (medium) did not lead to an increase in severity of colitis, but rather to a decrease of inflammation in the colon (average histological score: PBS, 2.5; LPS + IL-12 (10 ng), 1.0). No other marked pathological changes were observed in other organs (liver, lung) besides an occasional splenomegaly with and without infiltration of $CD4^+$ T cells (data not shown).

$CD4^+$ T cells from the skin of mice with psoriasis are $CD45Rb^{low}$ and produce high levels of IFN-γ and low levels of IL-4

To compare the activation/cytokine profile of SIL, we purified this population from the skin lesions of diseased and undiseased mice. Isolated SIL were stimulated in vitro with anti-CD3 and anti-CD28 for 48 h, and supernatants were tested for the production of IFN-γ, TNF-α, and IL-4. Lymphocytes isolated from the skin of mice that received T cells but showed no clinical signs of disease did not secrete any detectable levels of IFN-γ or IL-4. In contrast, cells from diseased mice expressed very high levels of IFN-γ and TNF-α and low levels of IL-4 (Table II). This pattern of cytokine expression was confirmed by intracellular cytokine staining (data not shown). Naive $CD4^+CD45Rb^{high}$ donor cells from spleens of BALB/c were stimulated in a similar fashion and showed no detectable levels of any cytokine tested. Furthermore, the majority of $CD4^+$ cells isolated from the inflamed tissue of diseased mice were $CD45Rb^{low}$ (Fig. 2). The data suggest that the majority of naive T cells transferred into *scid/scid* mice differentiate in the microenvironment of the skin into Th1-like memory/effector T cells.

IFN-γ-deficient $CD4^+CD45Rb^{high}$ T cells are able to induce psoriasiform lesions in scid *mice.*

To examine whether IFN-γ has a primary role in the induction of psoriasiform lesions, we first transferred naive T cells from IFN-γ-deficient ($IFN\text{-}\gamma^{-/-}$) mice into C.B-17 *scid/scid* mice. Interestingly, we found that despite the lack of IFN-γ, $CD4^+CD45Rb^{high}$ T cells from $IFN\text{-}\gamma^{-/-}$ donors were able to induce psoriasiform lesions in *scid/scid* mice (Fig. 3). Disease induction occurred with similar frequency, but ear thickness in diseased $IFN\text{-}\gamma^{-/-}$ T cell *scid/scid* mice was, on average, less than in control mice, and skin lesions on eyes and face were present but were less pronounced. The average clinical score of the diseased mice was 0.9 ± 1.0, and only one case of severe psoriasis (clinical score ≥ 3) was noted (see Table IV). In addition, disease onset was delayed (10–12 wk after cell transfer), when compared with control mice (average of 6–8 wk after cell transfer). Consistent with these observations, it appeared that in particular the hyperkeratosis in the skin of $IFN\text{-}\gamma^{-/-}$ T cell *scid/scid* mice was less pronounced (Fig. 1*G*).

The absence of donor-derived IFN-γ production was verified by testing the supernatants of isolated lymphocytes from the skin of diseased $CD4^+CD45Rb^{high}$ $IFN\text{-}\gamma^{-/-}$ reconstituted *scid/scid* mice after 48 h of stimulation with anti-CD3 and anti-CD28. No detectable levels of IFN-γ (≤30 pg) were found in any of the samples when tested by ELISA (Table III). The expression of TNF-α was also measured and found to be elevated but significantly less than the TNF-α levels observed in mice reconstituted with $CD4^+CD45Rb^{high}$ T cells from wild-type animals (Table III).

To further rule out that minute levels of IFN-γ secreted by host NK cells are sufficient to induce disease, we injected $IFN\text{-}\gamma^{-/-}$ T cells into *scid/beige* mice. These mice carry in addition to the *scid* mutations, the *beige* mutation that causes a deficiency in NK cells in addition to the T and B cell deficiency already present in the *scid* mutation. As shown in Fig. 1*H*, *scid/beige* mice that received $IFN\text{-}\gamma^{-/-}$ $CD4^+CD45Rb^{high}$ T cells also developed a very significant increase in ear thickness; however, again, the onset of disease was significantly delayed (Fig. 3) and the incidence of disease was reduced (Table IV) when compared with mice that had received $IFN\text{-}\gamma^{+/+}$ $CD4^+CD45Rb^{high}$ T cells. In addition, the severity of disease as measured by ear thickness (Fig. 3) and clinical score (Table IV) was attenuated. Interestingly, despite the presence of severe mononuclear cell infiltration, the acanthosis, consistent with above results, was less pronounced in these animals (Fig. 1*H*).

epidermal (micro)-abscess formation, and mononuclear lymphocyte infiltration (magnification, ×100). *E*, eyelid; the same hallmarks of psoriasis can also be seen here with the addition of elongated rete peg formations (i.e., down-growths of epidermis into dermis) (arrows) (magnification, ×100). *F*, Tail; note elongated rete pegs (arrows) as well as severe acanthosis with aggregations of neutrophils forming abscesses. Vessels are surrounded by mononuclear cells contain large numbers of marginated neutrophils (magnification, ×100). *G*, Tissue (ear) from *scid/scid* mice that received IFN-γ-deficient $CD4^+CD45Rb^{high}$ T cells and LPS plus IL-12 (10 ng). The acanthosis is less severe than in the scid/scid mice that received wild-type T cells (magnification, ×100). *H*, Tissue (ear) from *scid/beige* mice that received IFN-γ-deficient $CD4^+CD45Rb^{high}$ T cells and LPS plus IL-12 (10 ng). The acanthosis, but not the inflammation, is less severe than in the *scid/scid* mice that received wild-type T cells (magnification, ×100). *I*, Tissue (ear) from mice treated twice with anti-IL-12 (0.5 mg each time). The skin tissue is indistinguishable from sample shown in *A* (magnification, ×100). *J*, *scid/scid* mouse with severe psoriasis after $CD45Rb^{high}$ T cell transfer and administration of LPS (20 μg) and IL-12 (10 ng). The mouse shows extensive hair loss, severe erythema with ulceration and scaling with plaque-like elevations, especially on ear and face (clinical score 4).

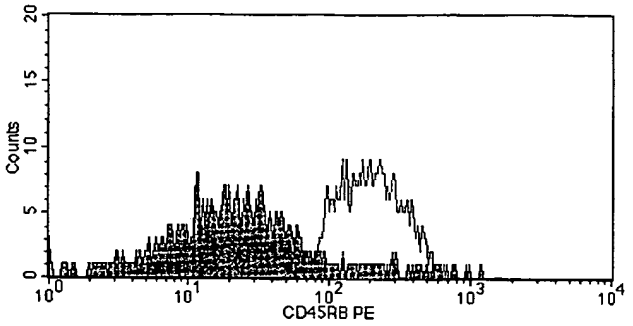

FIGURE 2. $CD4^+$ T cells isolated from psoriatic lesions express low levels of CD45Rb. Total cells from psoriasiform lesions were stained with APC-conjugated anti-CD4 and PE-conjugated CD45Rb. The histogram shows CD45 positive staining of gated $CD4^+$ cells. Donor cells (open histogram) are compared with cells that were isolated from psoriasiform lesions (filled histogram). Data are representative of at least four independent experiments.

These results indicate that IFN-γ regulates either directly or indirectly keratinocyte proliferation, but not mononuclear cell infiltration/activation in psoriasis.

IL-12 is highly expressed in psoriasiform lesions and in vivo neutralization of IL-12 down-regulates TNF-α and IFN-γ and inhibits disease development

We next focused on IL-12, since this proinflammatory cytokine plays a key role in the induction of IFN-γ. We performed immunohistochemical studies to detect the presence of heterodimeric IL-12 in inflamed tissue. The illustrations presented are anti-IL-12-PE stained 5-μm cross-sections from samples of ears of diseased mice. As shown in Fig. 4*A*, there is a very significant amount of IL-12 (p35/p40 (p70)) heterodimer expressed in the tissue of diseased $CD4^+CD45Rb^{high}$-treated mice. In contrast, significant less staining could be observed in $CD4^+CD45Rb^{high}$-treated animals that were injected with anti-IL-12 mAb (0.5 mg/mouse) on day 7 and 35 (Fig. 4*B*).

To further evaluate the role of IL-12 in the induction of psoriasiform lesions, we administered anti-IL-12 mAb (0.5 mg) at day 7 and 35 after T cell transfer to *scid/scid* mice that had received wild-type $CD4^+CD45Rb^{high}$ T cells. Two doses of anti-IL-12 mAb were given to maintain a high enough Ab titer over the entire period of disease induction after the transfer of $CD4^+CD45Rb^{high}$ T cells. In two independent experiments, mice (group of five) that were treated with anti-IL-12 mAb were completely protected from developing disease. Only 1 mouse out of 10 developed visible mild psoriasis in the form of slight hair loss and erythema around the eyelids (clinical score 1); however, this mouse and all other mice treated with anti-IL-12 mAb did not develop any increase in ear thickness (Fig. 3). In contrast, control mice that were treated with rat IgG control Abs or with PBS showed an incidence of 90% (9 of 10 mice developed disease) with an average clinical score of 2.4 ± 0.7 (Table IV). The higher incidence and severity of disease in this group was also associated with a significantly higher increase in ear thickness over time (Fig. 3).

The ears and skin of mice that received anti-IL-12 were examined for the cytokine production of infiltrating lymphocytes. While there were very few lymphocytes present in the skin of anti-IL-12 treated animals, these were isolated and tested for IFN-γ, IL-4, and TNF-α production. Only low levels of IFN-γ, IL-4, and TNF-α were detected in the supernatants of cells isolated from treated animals when compared with the cytokine production of supernatants obtained from control animals (Table III).

The results above are corroborated by the analysis of the histopathological sections obtained from animals that were treated with anti-IL-12 mAb. Mice that had been treated with with 0.5 mg of anti-IL-12 mAb on day 7 and 35 lacked any signs of significant inflammation, acanthosis or hyperkeratosis (Fig. 1*I*). Thus, anti-IL-12 administration seems to prevent the development of psoriasiform lesions by inhibiting keratinocyte hyperproliferation and mononuclear cell infiltration most likely by down-regulating both IFN-γ and TNF-α production.

Discussion

Psoriasis is a papulosquamous skin disease associated with rapid epidermal proliferation and a chronic mixed mononuclear cell infiltrate, including macrophages, dendritic cells, and activated T

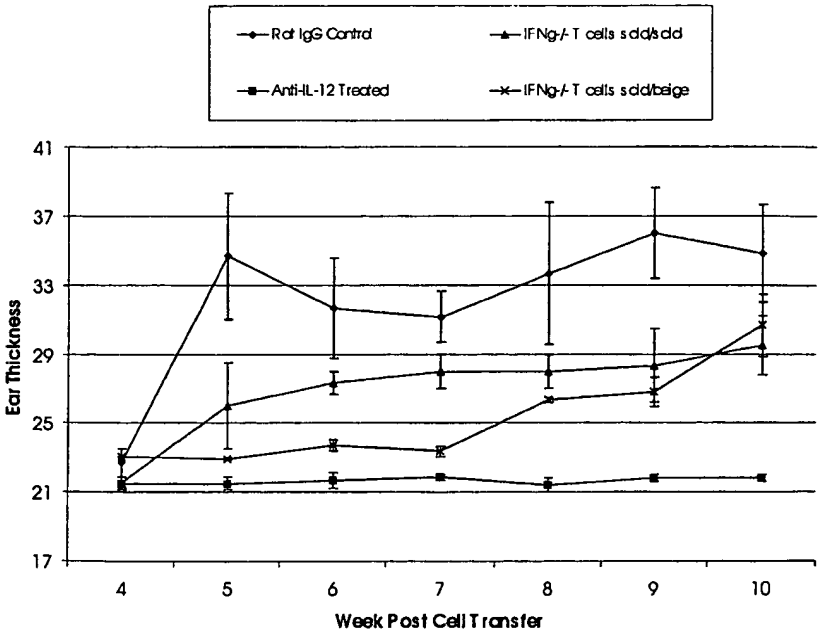

FIGURE 3. Administration of anti-IL-12, but not the transfer IFN-$\gamma^{-/-}$ $CD4^+CD45Rb^{high}$ T cells, inhibits the development of psoriatic skin lesions in *scid/scid* mice. C.B-17 *scid/scid* mice were reconstituted with 3×10^5 $CD4^+CD45Rb^{high}$ cells from the spleen of BALB/c mice and injected with 20 μg LPS on day 1 and twice with 10 ng IL-12 on day 1 and 3. Control mice received rat IgG Ab ($n = 5$). The experimental group was treated with anti-IL-12 mAb (clone C17.8 rat IgG1, PharMingen) on day 7 and 35 ($n = 5, p < 0.001$). In the IFN-$\gamma^{-/-}$ T cells transfer group either *scid/scid* ($n = 5, p < 0.005$) or *scid/beige* mice ($n = 7$, $p < 0.013$) received 3×10^5 $CD4^+CD45Rb^{high}$ from spleens of IFN-γ-deficient mice. Recipient mice were treated with LPS and IL-12 as described above. Ear thickness is measured in μm and is reported in weekly time intervals. Data represents the average ± SEM of four to five animals per group of one experiment. One additional experiment of the control, anti-IL-12 group, and the IFN-$\gamma^{-/-}$ group gave similar results. Statistical analysis was performed using the two-tailed Student's *t* test.

Table III. *Anti-IL-12 treatment reduces T cell inflammatory responses significantly, and IFN-$\gamma^{-/-}$ SIL produce TNF-α*[a]

T Cell Source	IFNγ (pg)	IL-4 (pg)	TNFα (pg)
Control mice[b]	56,380 ± 7,940	76 ± 5	698 ± 195
Anti-IL-12-treated mice[c]	300 ± 7	46 ± 4.1	≤35
Mice reconstituted with $CD4^+CD45RB^{high}$IFN-$\gamma^{-/-}$ BALB/c T cells[d]	≤20	69 ± 7.3	122 ± 31

[a] IFN-γ, IL-4, and TNF-α production by 2.0×10^5 SIL was measured as described in *Materials and Methods*. Data represent the mean ± SD. All mice were sacrificed at week 10 after T cell transfer. Control and treatment groups consisted of four to five mice. Lymphocytes from each group were pooled for in vitro stimulation regardless of disease status.

[b] Cells were isolated from psoriasiform lesions of 5–10 diseased *scid/scid* mice following reconstitution with $CD4^+CD45Rb^{high}$ and LPS + IL-12 administration. Mice did not receive any treatment. Data are from one experiment representative of three independent experiments showing similar values.

[c] Mice were treated twice i.p. with 0.5 mg of anti-IL-12 mAb (clone C17.8 rat IgG1) on day 7 and 35. Mice were sacrificed at week 10 after T cell transfer. Data are from one experiment representative of two independent experiments showing similar values.

[d] Cells isolated from psoriatic skin from the ears and eyelids of recipient scid/scid mice that were reconstituted with $CD4^+CD45RB^{high}$ cell from BALB/c IFN-$\gamma^{-/-}$ mice.

cells. Although this histopathology together with reports that administration of Abs against CD4 or fragments of diphtheria toxin fused to IL-2 may be beneficial in patients with psoriasis (18, 49–52) suggest an important role of T cells in the pathogenesis of this disease, the precise function of such inflammatory T cells is still unknown. Recently, Schon et al. (15) provided direct evidence that T cells may play a central role in the pathogenesis of the disease, by demonstrating that the transfer of minor-haplotype mismatched naive T cells into *scid/scid* mice was able to induce psoriasiform lesions in these mice. In these experiments, the expression and time to induction of disease was highly dependent on the specific minor histocompatibility Ags expressed by the donor cells. For instance, when BALB/c donor cells were used, the psoriasiform lesions induced in *scid/scid* mice were typically mild. However, when using different donor cells from F_2 (BALB/c × 129/SvJ) resulting in a greater genetic difference between donor and recipient mice, the authors were able to demonstrate a significant increase in the severity of the lesions. Although these studies were the first to show that T cells transferred into a *scid/scid* mouse can be directly responsible for the induction of psoriasis, they reveal little about the role of exogenous stimuli and the pathogenic mechanisms of the T cell involvement in the disease process.

Thus, we first examined in the present study the question of how immunomodulatory stimuli, in this case microbial Ags and the proinflammatory lymphokine IL-12, effect the ability of T cells to induce psoriasis in this newly developed $CD4^+CD45Rb^{high}$ T cells transfer model. We show that coadministration of LPS and IL-12 (1 and 10 ng) led to a more rapid onset and to an increased incidence of psoriasis in C.B-17 *scid* mice. In addition, the observed lesions in treated mice were also more severe. In additional experiments, we were able to demonstrate that coadministration of SEB also led to a significant increase in disease incidence and expression. These findings are very intriguing in light of reports that a significant number of patients report bacterial or viral infections before the appearance of psoriasiform lesions (reviewed in Refs. 53–55) and in light of recent animal model data that have suggested a role of bacterial superantigens in the pathogenesis of psoriasis (17, 56). Most notably, the skin lesions that developed by this method of induction were characteristic and remarkably similar to human psoriatic lesions, exhibiting most clinical and histological hallmarks. The scaling and thickening of skin evident macroscopically was due to marked hyper, parakeratosis, and acanthosis. It is further documented in the microscopic appearance of elongated rete pegs, ulcer, and pustule formation, and in the often severe epidermal hyperplasia. The inflammatory cell infiltration was primarily mononuclear and composed of lymphocytes with fewer monocytes, macrophages, and plasma cells. Moreover, the majority of $CD4^+$ T cells isolated from psoriasiform lesions express low levels of CD45Rb, which is consistent with the fact that recent studies in humans found that T cells isolated psoriatic plaques exhibit a memory phenotype (57). The above described histological characteristics (elongated rete pegs, microabscesses, acanthosis, hyperplasia, and hyperkeratosis) clearly distinguish this model from classic cutaneous graft-versus-host disease

Table IV. *Incidence and clinical score of $CD4^+CD45Rb^{high}$ scid mice*

T Cell/Recipient[a]	Incidence[b]	Average Clinical Score[c]	Severe Disease[d]
IFN-$\gamma^{+/+}$ *scid/scid*[e]	9/10 (90%)	2.4 ± 0.7	5/10 (50%)
IFN-$\gamma^{+/+}$ *scid/scid* α-IL-12[f]	1/10 (10%)	0.1 ± 0.3	0/10 (0%)
IFN-$\gamma^{-/-}$ *scid/scid*[g]	5/9 (56%)	0.9 ± 1.0	1/9 (11%)
IFN-$\gamma^{-/-}$ *scid/beige*[g]	3/7 (43%)	1.0 ± 1.3	2/7 (29%)

[a] All mice were reconstituted with either IFN-$\gamma^{+/+}$ or IFN-$\gamma^{-/-}$ $CD4^+CD45Rb^{high}$ T cells and LPS + IL-12.

[b] Disease incidence is reported as number of mice with disease over 10 wk of time: criteria being ear thickness ≥26 μm or clinical score of ≥1.

[c] Average clinical score ± SD was assessed of all mice in the group as described in *Materials and Methods*. Values: p vs IFN-$\gamma^{+/+}$ *scid/scid*: anti-IL-12; $p < 0.0000001$; IFN-$\gamma^{-/-}$ *scid/scid*; $p < 0.003$; IFN-$\gamma^{-/-}$ *scid/beige*; $p < 0.01$. Statistical analysis was performed using the two-tailed Student's t test.

[d] Severe disease induction calculated as the number of animals that received an average histology score of ≥2.5 and/or a clinical score of ≥3.

[e] Mice received either PBS or control Ab (rat IgG1).

[f] Ab treatment is described in *Materials and Method* and Table 3.

[g] *scid/scid* and *scid/beige* mice that were reconstituted with $CD4^+CD45Rb^{high}$ cell from BALB/c IFN-$\gamma^{-/-}$ mice.

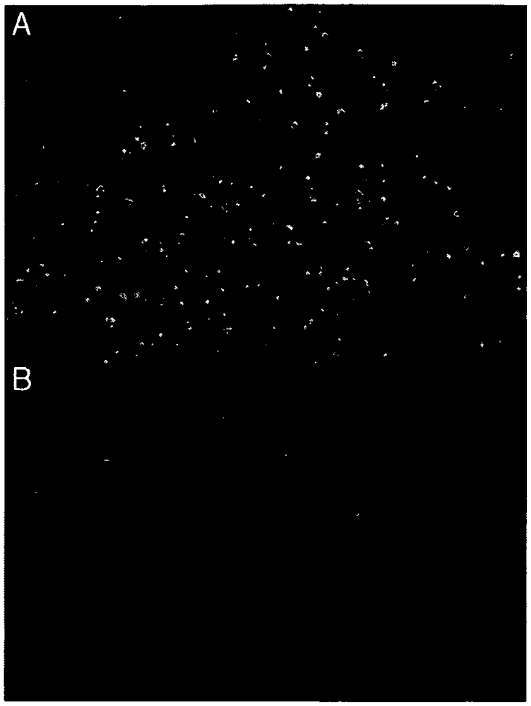

FIGURE 4. Immunohistochemical identification of heterodimeric IL-12 in the skin of *scid/scid* mice with psoriasis. *A*, High staining intensity of IL-12 heterodimer is seen in the skin of mice that were treated with control Ab (rat IgG). *B*, Few cells stain for IL-12 heterodimer in the skin of *scid/scid* mice that were treated twice i.p. with 0.5 mg of anti-IL-12 mAb (clone C17.8 rat IgG1) on day 7 and 35. Samples were taken 10 wk after T cell transfer and LPS and IL-12 (10 ng) administration. Tissue was stained with anti-IL-12 PE conjugated mAb (clone C17.8 rat IgG1; PharMingen) and photographed at ×200 magnification, using Kodak 400 film.

(GVHD), despite the fact that by definition through the use of minor haplotype mismatched T cells transfer this model could be interpreted as a GVHD model. Moreover, in contrast to psoriatic lesions, the histology of GVHD skin samples exhibits degenerating or necrotic keratinocytes, apoptotic basal cells surrounded by lymphocyte and dense dermal fibrosis (58), clearly not observed in this model. In fact, this and the SCID-hu xenogeneic transplantation model (17) are the only murine models of psoriasis to our knowledge in which the skin histology shows elongated rete pegs formation. Beside the similarity to the human histology observed in this study, there are also some differences. Most notable is the absence of $CD8^+$ T cells in this model which can be found in the epidermis of psoriatic plaques in humans (59–61). So far, however, a primary role for $CD8^+$ T cells in the pathophysiology of psoriasis has not been identified and successful initial therapies targeting $CD4^+$ T cells rather than $CD8^+$ T cells point toward the $CD4^+$ T cells as the primary culprit of the disease (49–52). Furthermore, the amount of $CD8^+$ T cells seems to also vary significantly in different stages and types of psoriasis (60, 62). Nevertheless, further experiments have to address the role of $CD8^+$ T cells in the $CD4^+$ T cell transfer model.

Several mechanisms could be responsible for the disease-promoting effects of LPS, IL-12, and SEB. First, these immunomodulators may assist in the proliferation and differentiation of naive Th0 cells to Th1 cells. This is quite plausible, since IL-12 alone without TNF-α or IL-1 fails to activate T cells (63–65) and microbial products; in particular, LPS can induce the production of these proinflammatory lymphokines by macrophages. In fact, recent studies have shown that microbial products, such as LPS, can directly stimulate TNF-α, IL-6, and to a lesser extent IL-12 production by murine skin-derived dendritic cells (66), and thus may be responsible for setting the proinflammatory condition for autoreactive T cells. Recent studies by Segal et al. (37) demonstrate that microbial products (LPS, *Escherichia coli* DNA, CpG oligonucleotides) can directly activate dormant myelin-basic protein-specific T cells into effector cells capable of inducing murine encephalitis, an effect that is dependent on IL-12. Thus, it is possible that $CD45Rb^{high}$ T cells after the transfer into SCID mice require additional IL-12 dependent signals to develop into autoimmune effector cells.

In addition to their immunostimulatory effects on macrophages and their effects on the development of Th1 effector cells, LPS, IL-12, and SEB may also modulate leukocyte trafficking in recipient mice to result in the cutaneous localization of Th1 effector cells. LPS promotes leukocyte recruitment by stimulating endothelial cell expression of E-selectin, ICAM-1, and VCAM-1 adhesion molecules both directly and through its ability to induce the production of IL-1, TNF-α and IFN-γ (reviewed in Refs. 67 and 68). The proinflammatory effects of these cytokines on leukocyte adhesion and migration is also well-known (67, 68). Most interestingly, in humans IL-12 and bacterial superantigens, such as SEB, have been demonstrated to induce the expression of the cutaneous lymphocyte Ag (CLA) on activated T cells (69). CLA^+ T cells are highly enriched in chronic inflammatory skin disorders and CLA appears to function as a homing receptor for the skin as it is a ligand for endothelial cell E-selectin (70–72). Additional experiments will address the relationship of these observations in humans to our studies in mice.

IFN-γ and IL-12 are two very important immunoregulatory cytokines that have been shown to play an important role in the development of autoimmune disorders (43, 73). IL-12 primarily activates NK and T cells, whereas IFN-γ primarily activates macrophages and induces the up-regulation of class II molecules on tissue cells. While the key function of IL-12 is the induction and maintenance of IFN-γ production in T cells during an immune response and in various autoimmune conditions, the role of IFN-γ during such processes, in particular whether IFN-γ is necessary for the IL-12 mediated generation of autoreactive inflammatory Th1 cells, has been controversial (36). Considering the presence of IFN-γ in psoriatic plaques in humans as well as in animal models and its putative involvement in the epithelial and keratinocyte abnormalities observed in patients (74–85) and our findings that IFN-γ is produced at very high levels by inflammatory T cells isolated directly from the lesions of psoriatic mice, one might have expected IFN-γ to be crucial for the induction of autoimmune effector cells in psoriasis. The data in this paper, however, indicate that IFN-γ may only participate in the disease process by enhancing disease severity, most likely by promoting keratinocyte proliferation, but clearly not by inducing and maintaining pathogenic, inflammatory T cells in psoriatic skin. This finding is supported by the fact that the histology observed in lesions of mice that received IFN-$\gamma^{-/-}$ donor T cells showed slightly lesser or equal signs of inflammation but hyperkeratosis or acanthosis was clearly diminished in *scid/scid* or *scid/beige* mice. Moreover, although clinical severity of disease, as measured by ear thickness and macroscopic observation, was attenuated when compared with control animals, the incidence of disease was very similar. This was also found to be true when IFN-$\gamma^{-/-}$ T cells were transferred into T, B, and NK cell-deficient *scid/beige* mice.

These results indicate that IFN-γ might not play a major role in the induction of chronic skin inflammation, but seems to be an important cofactor in the induction of aberrant keratinocyte proliferation. Such a notion is supported by the fact that the IFN-γ

receptor is present on keratinocytes (75) and by the work of Prinz et al. (46), in which they demonstrate that lesional psoriatic T lymphocytes are capable of promoting keratinocyte proliferation in vitro and that such mitogenic capacity can be inhibited by the addition of serum containing Abs against IFN-γ. Others have shown that IFN-γ is able to promote keratinocyte proliferation in the presence of psoriatic fibroblasts but not in the presence of healthy fibroblasts (83). In addition, it is possible that IFN-γ leads to keratinocyte hyperplasia by promoting keratinocyte survival through the induction of CD40 on the surface of keratinocytes (86) and/or through the induction of Bcl-X_L (87).

The absence of host-derived IFN-γ was verified by the measurement of IFN-γ from anti-CD3 and anti-CD28 stimulated whole cells isolated from the inflammatory lesions of animals that received IFN-$\gamma^{-/-}$ T cells. As expected, we were unable to detect any measurable level of IFN-γ, thus further ruling out the possibility that IFN-γ might be secreted by non-T cells in the skin, including NK cells. However, we cannot rule out that very small amounts of IFN-γ secreted by host cells could have inflammatory effects on donor T cells and host macrophages. Another possibility would be that in the IFN-$\gamma^{-/-}$ T cell/*scid* transfer experiment, Th2 inflammatory T cells are generated in the absence of IFN-γ that are capable of inducing psoriasiform lesions. That such immune deviation can cause disease has been recently reported in other autoimmune models that are classically associated with Th1-type inflammatory responses (88, 89). However, our data do not support this, since we were unable to detect elevated amounts of IL-4 in the supernatants of cells isolated from diseased IFN-$\gamma^{-/-}$ T cell/*scid* animals.

In contrast to IFN-γ, an absolute requirement for IL-12 in the development of chronic psoriasiform lesions in *scid/scid* mice was demonstrated by several observations made in our studies. First, medium and low doses of IL-12 (1 and 10 ng/mouse) administered following donor T cell transfer resulted in a higher incidence and severity of disease. Moreover, in situ staining of inflamed tissue revealed a significant present of heterodimeric IL-12 (p70), while IL-12 staining was not present at all in noninflamed control tissue. Most importantly, in vivo neutralization of IL-12 with a mAb reacting against IL-12 p70 heterodimer 7 days following T cell transfer was able to completely abrogate disease induction. An interesting aspect of our studies was that high doses of IL-12 (100 ng/mouse) actually inhibited disease induction instead of promoting disease development. This dose-dependent effect of IL-12 found in our studies are reminiscent of findings made by others in an animal model of rheumatoid arthritis (90). In this model, high doses of IL-12 for 3 wk successfully suppressed the induction of collagen induced arthritis in DBA/1 mice, while lower doses of IL-12 resulted in a more severe form of arthritis in these mice (90, 91). The reasons for these opposing effects are currently unknown.

Although our finding of the IFN-γ-independent effect of IL-12 in the generation of inflammatory T cells during the onset of psoriasis are quite surprising, they are not entirely unexpected. Very recently, others have provided evidence, that IFN-γ under some condition may not be crucial for the induction of other autoimmune conditions as well. For example, Simpson et al. (92) demonstrated that IFN-$\gamma^{-/-}$ T cell reconstituted animals developed colitis and wasting disease at a similar rate and severity as IFN-$\gamma^{+/+}$ cell reconstituted mice. In more recent studies, Davidson et al. (93) demonstrated very nicely that IFN-γ seems to be only important during the onset (acute) phase of colitis, since the administration of anti-IFN-γ mAb prevented disease onset, but neutralization of IFN-γ during the chronic phase had no effect on reversing colitis in IL-10 KO mice. Moreover, Segal et al. (94) showed that IL-12 is able to induce experimental allergic encephalitis (EAE) in the presence or absence of IFN-γ. In an infectious disease model, IL-12 was able to exert antimicrobial activity against *Leishmania donovani* in IFN-$\gamma^{-/-}$ mice (95). Interestingly, the leishmanicidal activity of IL-12 was dependent on TNF-α and required the activity of inducible NO synthase. Since we were able to find high levels of TNF-α in the supernatants of cells extracted from the IFN-$\gamma^{-/-}$ T cell transfer *scid* animals, it is possible that the IL-12 disease-inducing effects in our model are also TNF-α dependent. Although our findings and above considerations point strongly toward a Th1-mediated disease mechanism, the presence of substantial amounts of IL-4 in the supernatants of cells isolated from psoriasiform lesions justify a more close look at the role of this Th2 cytokine in the disease process. It is quite possible that the pathogenesis of psoriasiform lesions in this model is dependent on both Th1 and Th2 cytokine, most likely at different stages of the disease process.

In summary, the murine chronic skin disorder described in this study included features that are normally only observed in human psoriasis, such as rete pegs, severe acanthosis, and infiltration of Th1 cells into the dermis. The clinical and histopathological abnormalities were greatly enhanced by the in vivo administration of LPS and IL-12, suggesting an important role of infectious agent(s) in the pathogenesis of the disease. Moreover, we demonstrated for the first time that the induction of psoriasiform lesions was dependent on IL-12, but independent on IFN-γ. Thus, this study offers further insight into the specific pathogenic requirements of Th1 promoting cytokines and cells for the development of psoriasiform lesions and hopefully will provide further insight into the prevention and treatment of psoriasis in humans.

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References

1. Dahl, M. V. 1996. *Immunodermatology*. Mosby, St. Louis. 3rd Ed., pp. 301–315.
2. Christophers, E. 1996. The immunopathology of psoriasis. *Int. Arch. Allergy Immunol. 110:199.*
3. Barker, J. N. 1994. The immunopathology of psoriasis. *Baillieres Clin. Rheumatol. 8:429.*
4. Boehncke, W. H. 1996. Psoriasis and bacterial superantigens: formal or causal correlation? *Trends Microbiol. 4:485.*
5. Prinz, J. C. 1997. Psoriasis vulgaris, streptococci and the immune system: a riddle to be solved soon? *Scand. J. Immunol. 45:583.*
6. Rook, G. A., and J. L. Stanford. 1992. Slow bacterial infections or autoimmunity? *Immunol. Today. 13:160.*
7. Rosenberg, E. W., P. W. Noah, and R. B. Skinner, Jr. 1994. Microorganisms and psoriasis. *J. Natl. Med. Assoc. 86:305.*
8. Carroll, J. M., M. R. Romero, and F. M. Watt. 1995. Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. *Cell 83:957.*
9. Groves, R. W., H. Mizutani, J. D. Kieffer, and T. S. Kupper. 1995. Inflammatory skin disease in transgenic mice that express high levels of interleukin 1α in basal epidermis. *Proc. Natl. Acad. Sci. USA 92:11874.*
10. Wilson, J. B., W. Weinberg, R. Johnson, S. Yuspa, and A. J. Levine. 1990. Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. *Cell 61:1315.*
11. Yanagisawa, H., J. A. Richardson, J. D. Taurog, and R. E. Hammer. 1995. Characterization of psoriasiform and alopecic skin lesions in HLA-B27 transgenic rats. *Am. J. Pathol. 147:955.*
12. Bullard, D. C., K. Scharffetter-Kochanek, M. J. McArthur, J. G. Chosay, M. E. McBride, C. A. Montgomery, and A. L. Beaudet. 1996. A polygenic mouse model of psoriasiform skin disease in CD18-deficient mice. *Proc. Natl. Acad. Sci. USA 93:2116.*
13. Nanney, L. B., J. P. Sundberg, and L. E. King. 1996. Increased epidermal growth factor receptor in *fsn/fsn* mice. *J. Invest. Dermatol. 106:1169.*

14. Sundberg, J. P., M. France, D. Boggess, B. A. Sundberg, A. B. Jenson, W. G. Beamer, and L. D. Shultz. 1997. Development and progression of psoriasiform dermatitis and systemic lesions in the flaky skin (fsn) mouse mutant. *Pathobiology 65:271.*
15. Schon, M. P., M. Detmar, and C. M. Parker. 1997. Murine psoriasis-like disorder induced by naive $CD4^+$ T cells. *Nat. Med. 3:183.*
16. Nickoloff, B. J., S. L. Kunkel, M. Burdick, and R. M. Strieter. 1995. Severe combined immunodeficiency mouse and human psoriatic skin chimeras: validation of a new animal model. *Am. J. Pathol. 146:580.*
17. Wrone-Smith, T., and B. J. Nickoloff. 1996. Dermal injection of immunocytes induces psoriasis. *J. Clin. Invest. 98:1878.*
18. Gottlieb, S. L., P. Gilleaudeau, R. Johnson, L. Estes, T. G. Woodworth, A. B. Gottlieb, and J. G. Krueger. 1995. Response of psoriasis to a lymphocyte-selective toxin (DAB389IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. *Nat. Med. 1:442.*
19. Podolsky, D. K. 1991. Inflammatory bowel disease (1). *N. Engl. J. Med. 325:928.*
20. Taurog, J. D. 1994. The germfree state prevents development of gut and joint inflammatory disease in HLAB2 transgenic rats. *J. Exp. Med. 180:2359.*
21. Ehrhardt, R. O., B. R. Ludviksson, B. Gray, M. Neurath, and W. Strober. 1997. Induction and prevention of colonic inflammation in IL-2-deficient mice. *J Immunol. 158:566.*
22. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell 75:263.*
23. Mombaerts, P., E. Mizoguchi, M. J. Grusby, L. H. Glimcher, A. K. Bhan, and S. Tonegawa. 1993. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell 75:274.*
24. Schultz, M., R. Sellon, S. Tonkonogy, E. Balish, and R. Sartor. 1997. IL-2 deficient mice raised under germ-free conditions develop delayed mild focal intestinal inflammation and progressive loss of B cells. *Gastroenterology 112:1086.*
25. Aranda, R., B. C. Sydora, P. L. McAllister, S. W. Binder, H. Y. Yang, S. R. Targan, and M. Kronenberg. 1997. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of $CD4^+$, $CD45RB^{high}$ T cells to SCID recipients. *J Immunol. 158:3464.*
26. Rath, H. C., H. H. Herfarth, J. S. Ikeda, W. B. Grenther, T. E. Hamm, Jr., E. Balish, J. D. Taurog, R. E. Hammer, K. H. Wilson, and R. B. Sartor. 1996. Normal luminal bacteria, especially *Bacteroides* species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human β_2 microglobulin transgenic rats. *J Clin Invest. 98:945.*
27. Shomer, N. H., C. A. Dangler, M. D. Schrenzel, and J. G. Fox. 1997. *Helicobacter* bilis-induced inflammatory bowel disease in *scid* mice with defined flora. *Infect. Immun. 65:4858.*
28. Cahill, R. J., C. J. Foltz, J. G. Fox, C. A. Dangler, F. Powrie, and D. B. Schauer. 1997. Inflammatory bowel disease: an immunity-mediated condition triggered by bacterial infection with *Helicobacter* hepaticus. *Infect. Immun. 65:3126.*
29. Finn, A., S. Strobel, M. Levin, and N. Klein. 1994. Endotoxin-induced neutrophil adherence to endothelium: relationship to CD11b/CD18 and L-selectin expression and matrix disruption. *Ann. NY Acad. Sci. 725:173.*
30. Lynam, E. B., S. I. Simon, Y. P. Rochon, and L. A. Sklar. 1994. Lipopolysaccharide enhances CD11b/CD18 function but inhibits neutrophil aggregation. *Blood 83:3303.*
31. Crockett-Torabi, E., B. Sulenbarger, C. W. Smith, and J. C. Fantone. 1995. Activation of human neutrophils through L-selectin and Mac-1 molecules. *J. Immunol. 154:2291.*
32. Spertini, O., F. W. Luscinskas, G. S. Kansas, J. M. Munro, J. D. Griffin, M. A. Gimbrone, Jr., and T. F. Tedder. 1991. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. *J. Immunol. 147:2565.*
33. Neumann, B., B. Engelhardt, H. Wagner, and B. Holzmann. 1997. Induction of acute inflammatory lung injury by staphylococcal enterotoxin B. *J. Immunol. 158:1862.*
34. Krakauer, T. 1994. Cell adhesion molecules are co-receptors for staphylococcal enterotoxin B-induced T-cell activation and cytokine production. *Immunol. Lett. 39:121.*
35. Mehindate, K., R. al-Daccak, F. Damdoumi, and W. Mourad. 1996. Synergistic effect between CD40 and class II signals overcome the requirement for class II dimerization in superantigen-induced cytokine gene expression. *Eur. J. Immunol. 26:2075.*
36. Trinchieri, G. 1998. Proinflammatory and immunoregulatory functions of interleukin-12. *Int. Rev. Immunol. 16:365.*
37. Segal, B. M., D. M. Klinman, and E. M. Shevach. 1997. Microbial products induce autoimmune disease by an IL-12-dependent pathway. *J. Immunol. 158:5087.*
38. Neurath, M. F., I. Fuss, B. L. Kelsall, E. Stuber, and W. Strober. 1995. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J. Exp. Med. 182:1281.*
39. Tarrant, T. K., P. B. Silver, C. C. Chan, B. Wiggert, and R. R. Caspi. 1998. Endogenous IL-12 is required for induction and expression of experimental autoimmune uveitis. *J. Immunol. 161:122.*
40. Gately, M. K., L. M. Renzetti, J. Magram, A. S. Stern, L. Adorini, U. Gubler, and D. H. Presky. 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu. Rev. Immunol. 16:495.*
41. Okura, Y., K. Takeda, S. Honda, H. Hanawa, H. Watanabe, M. Kodama, T. Izumi, Y. Aizawa, S. Seki, and T. Abo. 1998. Recombinant murine interleukin-12 facilitates induction of cardiac myosin-specific type 1 helper T cells in rats. *Circ. Res. 82:1035.*
42. Trembleau, S., T. Germann, M. K. Gately, and L. Adorini. 1995. The role of IL-12 in the induction of organ-specific autoimmune diseases. *Immunol. Today 16:383.*
43. Billiau, A. 1996. Interferon-γ in autoimmunity. *Cytokine Growth Factor Rev. 7:25.*
44. Vollmer, S., A. Menssen, P. Trommler, D. Schendel, and J. C. Prinz. 1994. T lymphocytes derived from skin lesions of patients with psoriasis vulgaris express a novel cytokine pattern that is distinct from that of T helper type 1 and T helper type 2 cells. *Eur. J. Immunol. 24:2377.*
45. Schlaak, J. F., M. Buslau, W. Jochum, E. Hermann, M. Girndt, H. Gallati, K. H. Meyer zum Buschenfelde, and B. Fleischer. 1994. T cells involved in psoriasis vulgaris belong to the Th1 subset. *J. Invest. Dermatol. 102:145.*
46. Prinz, J. C., B. Gross, S. Vollmer, P. Trommler, I. Strobel, M. Meurer, and G. Plewig. 1994. T cell clones from psoriasis skin lesions can promote keratinocyte proliferation in vitro via secreted products. *Eur. J. Immunol. 24:593.*
47. Romagnani, P., F. Annunziato, M. C. Baccari, and P. Parronchi. 1997. T cells and cytokines in Crohn's disease. *Curr. Opin. Immunol. 9:793.*
48. Nickoloff, B. J., and T. Wrone-Smith. 1997. Animal models of psoriasis. *Nat. Med. 3:475.*
49. Nicolas, J. F., N. Chamchick, J. Thivolet, J. Wijdenes, P. Morel, and J. P. Revillard. 1991. CD4 antibody treatment of severe psoriasis. *Lancet 338:321.*
50. Prinz, J., O. Braun-Falco, M. Meurer, P. Daddona, C. Reiter, P. Rieber, and G. Riethmuller. 1991. Chimaeric CD4 monoclonal antibody in treatment of generalised pustular psoriasis. *Lancet 338:320.*
51. Rizova, H., J. F. Nicolas, P. Morel, J. Kanitakis, A. Demidem, J. P. Revillard, J. Wijdenes, J. Thivolet, and D. Schmitt. 1994. The effect of anti-CD4 monoclonal antibody treatment on immunopathological changes in psoriatic skin. *J. Dermatol. Sci. 7:1.*
52. Morel, P., J. P. Revillard, J. F. Nicolas, J. Wijdenes, H. Rizova, and J. Thivolet. 1992. Anti-CD4 monoclonal antibody therapy in severe psoriasis. *J. Autoimmun. 5:465.*
53. Schafer, R., and J. M. Sheil. 1995. Superantigens and their role in infectious disease. *Adv. Pediatr. Infect. Dis. 10:369.*
54. Valdimarsson, H., H. Sigmundsdottir, and I. Jonsdottir. 1997. Is psoriasis induced by streptococcal superantigens and maintained by M-protein-specific T cells that cross-react with keratin? *Clin. Exp. Immunol. 107(Suppl. 1):21.*
55. Tagami, H. 1997. Triggering factors. *Clin. Dermatol. 15:677.*
56. Boehncke, W. H., T. M. Zollner, D. Dressel, and R. Kaufmann. 1997. Induction of psoriasiform inflammation by a bacterial superantigen in the SCID-hu xenogeneic transplantation model. *J. Cutan. Pathol. 24:1.*
57. Prens, E., R. Debets, and J. Hegmans. 1995. T lymphocytes in psoriasis. *Clin Dermatol. 13:115.*
58. Christofidou-Solomidou, M., S. M. Albelda, F. C. Bennett, and G. F. Murphy. 1997. Experimental production and modulation of human cytotoxic dermatitis in human-murine chimeras. *Am. J. Pathol. 150:631.*
59. Austin, L. M., T. R. Coven, N. Bhardwaj, R. Steinman, and J. G. Krueger. 1998. Intraepidermal lymphocytes in psoriatic lesions are activated GMP-17(TIA-1)$^+$$CD8^+$$CD3^+$ CTLs as determined by phenotypic analysis. *J. Cutan. Pathol. 25:79.*
60. Onuma, S. 1994. Immunohistochemical studies of infiltrating cells in early and chronic lesions of psoriasis. *J. Dermatol. 21:223.*
61. Krueger, J. G., J. T. Wolfe, R. T. Nabeya, V. P. Vallat, P. Gilleaudeau, N. S. Heftler, L. M. Austin, and A. B. Gottlieb. 1995. Successful ultraviolet B treatment of psoriasis is accompanied by a reversal of keratinocyte pathology and by selective depletion of intraepidermal T cells. *J. Exp. Med. 182:2057.*
62. Baker, B. S., A. V. Powles, S. Lambert, H. Valdimarsson, and L. Fry. 1988. A prospective study of the Koebner reaction and T lymphocytes in uninvolved psoriatic skin. *Acta Derm. Venereol. 68:430.*
63. D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J. Exp. Med. 178:1041.*
64. Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher. 1993. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon γ by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc. Natl. Acad. Sci. USA 90:6115.*
65. Hunter, C. A., R. Chizzonite, and J. S. Remington. 1995. IL-1 beta is required for IL-12 to induce production of IFN-γ by NK cells: a role for IL-1β in the T cell-independent mechanism of resistance against intracellular pathogens. *J. Immunol. 155:4347.*
66. Jacob, T., P. S. Walker, A. M. Krieg, M. C. Udey, and J. C. Vogel. 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol. 161:3042.*
67. Pober, J. S., and R. S. Cotran. 1990. Cytokines and endothelial cell biology. *Physiol. Rev. 70:427.*
68. Bevilacqua, M. P. 1993. Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol. 11:767.*
69. Leung, D. Y., M. Gately, A. Trumble, B. Ferguson-Darnell, P. M. Schlievert, and L. J. Picker. 1995. Bacterial superantigens induce T cell expression of the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen, via stimulation of interleukin 12 production. *J. Exp. Med. 181:747.*
70. Picker, L. J., S. A. Michie, L. S. Rott, and E. C. Butcher. 1990. A unique phenotype of skin-associated lymphocytes in humans: preferential expression of the HECA-452 epitope by benign and malignant T cells at cutaneous sites. *Am. J. Pathol. 136:1053.*

71. Picker, L. J., T. K. Kishimoto, C. W. Smith, R. A. Warnock, and E. C. Butcher. 1991. ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature 349:796.*
72. Berg, E. L., T. Yoshino, L. S. Rott, M. K. Robinson, R. A. Warnock, T. K. Kishimoto, L. J. Picker, and E. C. Butcher. 1991. The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. *J. Exp. Med. 174:1461.*
73. Schattner, A. 1994. Lymphokines in autoimmunity: a critical review. *Clin. Immunol. Immunopathol. 70:177.*
74. Barna, M., F. G. Snijdewint, F. L. van der Heijden, J. D. Bos, and M. L. Kapsenberg. 1994. Characterization of lesional psoriatic skin T lymphocyte clones. *Acta Derm. Venereol. Suppl. 186:9.*
75. Scheynius, A., J. Fransson, C. Johansson, H. Hammar, B. Baker, L. Fry, and H. Valdimarsson. 1992. Expression of interferon-γ receptors in normal and psoriatic skin. *J. Invest. Dermatol. 98:255.*
76. Yamamoto, T., M. Matsuuchi, I. Katayama, and K. Nishioka. 1998. Repeated subcutaneous injection of staphylococcal enterotoxin B- stimulated lymphocytes retains epidermal thickness of psoriatic skin-graft onto severe combined immunodeficient mice. *J. Dermatol. Sci. 17:8.*
77. Lemster, B. H., P. B. Carroll, H. R. Rilo, N. Johnson, A. Nikaein, and A. W. Thomson. 1995. IL-8/IL-8 receptor expression in psoriasis and the response to systemic tacrolimus (FK506) therapy. *Clin. Exp. Immunol. 99:148.*
78. Gearing, A. J., N. J. Fincham, C. R. Bird, M. Wadhwa, A. Meager, J. E. Cartwright, and R. D. Camp. 1990. Cytokines in skin lesions of psoriasis. *Cytokine 2:68.*
79. Uyemura, K., M. Yamamura, D. F. Fivenson, R. L. Modlin, and B. J. Nickoloff. 1993. The cytokine network in lesional and lesion-free psoriatic skin is characterized by a T-helper type 1 cell-mediated response. *J. Invest. Dermatol. 101:701.*
80. Nestle, F. O., L. A. Turka, and B. J. Nickoloff. 1994. Characterization of dermal dendritic cells in psoriasis: Autostimulation of T lymphocytes and induction of Th1 type cytokines. *J. Clin. Invest. 94:202.*
81. Sigmundsdottir, H., B. Sigurgeirsson, M. Troye-Blomberg, M. F. Good, H. Valdimarsson, and I. Jonsdottir. 1997. Circulating T cells of patients with active psoriasis respond to streptococcal M-peptides sharing sequences with human epidermal keratins. *Scand. J. Immunol. 45:688.*
82. Saunders, N., A. Dahler, S. Jones, R. Smith, and A. Jetten. 1996. Interferon-γ as a regulator of squamous differentiation. *J. Dermatol. Sci. 13:98.*
83. Fransson, J., Q. Shen, A. Scheynius, and H. Hammar. 1996. The effect of IFN-γ on healthy and psoriatic keratinocytes in a skin equivalent model is influenced by the source of the keratinocytes and by their interactions with fibroblasts. *Arch. Dermatol. Res. 289:14.*
84. Kaneko, F., M. Suzuki, Y. Takiguchi, N. Itoh, and T. Minagawa. 1990. Immunohistopathologic studies in the development of psoriatic lesion influenced by γ-interferon and the producing cells. *J. Dermatol. Sci. 1:425.*
85. Nickoloff, B. J., R. S. Mitra, J. T. Elder, G. J. Fisher, and J. J. Voorhees. 1989. Decreased growth inhibition by recombinant γ interferon is associated with increased transforming growth factor-α production in keratinocytes cultured from psoriatic lesions. *Br. J. Dermatol. 121:161.*
86. Denfeld, R. W., D. Hollenbaugh, A. Fehrenbach, J. M. Weiss, A. von Leoprechting, B. Mai, U. Voith, E. Schopf, A. Aruffo, and J. C. Simon. 1996. CD40 is functionally expressed on human keratinocytes. *Eur. J. Immunol. 26:2329.*
87. Wrone-Smith, T., T. Johnson, B. Nelson, L. H. Boise, C. B. Thompson, G. Nunez, and B. J. Nickoloff. 1995. Discordant expression of Bcl-x and Bcl-2 by keratinocytes in vitro and psoriatic keratinocytes in vivo. *Am. J. Pathol. 146: 1079.*
88. Lafaille, J. J., F. V. Keere, A. L. Hsu, J. L. Baron, W. Haas, C. S. Raine, and S. Tonegawa. 1997. Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J. Exp. Med. 186:307.*
89. Pakala, S. V., M. O. Kurrer, and J. D. Katz. 1997. T helper 2 (Th2) T cells induce acute pancreatitis and diabetes in immune-compromised nonobese diabetic (NOD) mice. *J. Exp. Med. 186:299.*
90. Hess, H., M. K. Gately, E. Rude, E. Schmitt, J. Szeliga, and T. Germann. 1996. High doses of interleukin-12 inhibit the development of joint disease in DBA/1 mice immunized with type II collagen in complete Freund's adjuvant. *Eur. J. Immunol. 26:187.*
91. Germann, T., J. Szeliga, H. Hess, S. Storkel, F. J. Podlaski, M. K. Gately, E. Schmitt, and E. Rude. 1995. Administration of interleukin 12 in combination with type II collagen induces severe arthritis in DBA/1 mice. *Proc. Natl. Acad. Sci. USA 92:4823.*
92. Simpson, S. J., S. Shah, M. Comiskey, Y. P. de Jong, B. Wang, E. Mizoguchi, A. K. Bhan, and C. Terhorst. 1998. T cell-mediated pathology in two models of experimental colitis depends predominantly on the interleukin 12/signal transducer and activator of transcription (Stat)-4 pathway, but is not conditional on interferon γ expression by T cells. *J. Exp. Med. 187:1225.*
93. Davidson, N. J., S. A. Hudak, R. E. Lesley, S. Menon, M. W. Leach, and D. M. Rennick. 1998. IL-12, but not IFN-γ, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J. Immunol. 161:3143.*
94. Segal, B. M., B. K. Dwyer, and E. M. Shevach. 1998. An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. *J. Exp. Med. 187:537.*
95. Taylor, A. P., and H. W. Murray. 1997. Intracellular antimicrobial activity in the absence of interferon-γ: effect of interleukin-12 in experimental visceral leishmaniasis in interferon-γ gene-disrupted mice. *J. Exp. Med. 185:1231.*

Prevention of Diabetes in NOD Mice by Administration of Dendritic Cells Deficient in Nuclear Transcription Factor-κB Activity

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Abnormalities of dendritic cells (DCs) have been identified in type 1 diabetic patients and in nonobese diabetic (NOD) mice that are associated with augmented nuclear transcription factor (NF)-κB activity. An imbalance that favors development of the immunogenic DCs may predispose to the disease, and restoration of the balance by administration of DCs deficient in NF-κB activity may prevent diabetes. DCs propagated from NOD mouse bone marrow and treated with NF-κB–specific oligodeoxyribonucleotide (ODN) in vitro (NF-κB ODN DC) were assessed for efficacy in prevention of diabetes development in vivo. Gel shift assay with DC nuclear extracts confirmed specific inhibition of NF-κB DNA binding by NF-κB ODN. The costimulatory molecule expression, interleukin (IL)-12 production, and immunostimulatory capacity in presenting allo- and islet-associated antigens by NF-κB ODN DC were significantly suppressed. NF-κB ODN renders DCs resistant to lipopolysaccharide stimulation. Administration of 2×10^6 NF-κB ODN DCs into NOD mice aged 6–7 weeks effectively prevented the onset of diabetes. T-cells from pancreatic lymph nodes of NF-κB ODN DC–treated animals exhibited hyporesponsiveness to islet antigens with low production of interferon-γ and IL-2. These findings provide novel insights into the mechanisms of autoimmune diabetes and may lead to development of novel preventive strategies. *Diabetes* 52: 1976–1985, 2003

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APC, antigen-presenting cell; CTL, cytotoxic T-lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IκB, inhibitor of κB; IL, interleukin; iNOS, inducible nitric oxide synthetase; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; NF-κB, nuclear transcription factor-κB; ODN, oligodeoxyribonucleotide; TNF, tumor necrosis factor; TUNEL, transferase-mediated dUTP nick-end labeling.

Insulin-dependent diabetes (type 1 diabetes) is an autoimmune disease characterized by T-cell–mediated destruction of the pancreatic β-cells (1). The nature of immune dysregulation leading to β-cell destruction remains poorly understood, but it is clearly influenced by multiple genetic, environmental, and immunological factors. In particular, antigen-presenting cells (APCs) have a significant impact on T-cell differentiation by providing costimulatory signals and secreting the cytokine milieu at the time of T-cell priming (2). Dendritic cells (DCs) are professional APCs and control immune responses to either augment or reduce autoimmunity, which is related to their state of maturation (3,4). Mature DCs express high major histocompatibility complex (MHC) and costimulatory molecules, secrete interleukin (IL)-12, and stimulate vigorous T-helper 1 responses. In contrast, immature DCs expressing low costimulatory molecules induce antigen-specific hyporesponsiveness by triggering T-cell apoptosis (5) or differentiation of regulatory T-cells (6). Maturational and functional abnormalities of DCs have been demonstrated in humans with type 1 diabetes (7,8) and in nonobese diabetic (NOD) mice (9). Indeed, DCs are among the first cell populations detected within the islets during the onset of diabetes, and diabetes can be induced by an adoptive transfer of syngeneic DCs treated with tumor necrosis factor (TNF)-α in NOD mice (8,10).

The activation of nuclear transcription factor (NF)-κB is important in DC maturation and activation (11). Mature DCs express NF-κB, and mice lacking NF-κB complexes fail to develop myeloid-derived DCs (12,13). Many inducible genes that encode cytokines, chemokines, cell adhesion molecules, growth factors, costimulatory molecules, and immune receptors contain NF-κB binding sites in their promoters or enhancers (14,15). NF-κB is present in the cytoplasm and associated with the inhibitor of κB (IκB) as an inactive complex. Various external and internal signals, including cytokines, lipopolysaccharide (LPS), and mitogens, lead to the dissociation of the NF-κB/IκB complex by degrading IκB and allowing the nuclear translocation of free NF-κB (11). In the nucleus, NF-κB binds to a specific DNA motif and regulates transcription of target genes. The development of diabetes has been correlated with elevated levels of NF-κB activation and enhanced antigen-presentation function in DCs of NOD mice (16,17), due to

hyperactive IκB kinase (11). In addition, DCs in NOD mice show an inability to effectively elicit regulatory T-cell function and a propensity to secrete high levels of IL-12, an important proinflammatory cytokine that drives T-helper 1 responses (13). Therefore, an imbalance favoring development of immunogenic DCs, such as those with elevated NF-κB activity, might predispose to developing autoimmune diabetes (18), and restoration of the balance by administering DCs deficient in NF-κB activity might prevent the development of diabetes.

We constructed decoy double-stranded oligodeoxynucleotides (ODNs) containing a consensus of NF-κB binding sites that inhibit NF-κB activity (19). The data of this study demonstrated that NF-κB ODN specifically inhibited NF-κB DNA binding capacity and prevented DC maturation and antigen-presentation capacity. Administration of the DCs deficient in NF-κB activity prevented diabetes development in NOD mice, which was associated with T-cell hyporesponsiveness to islet antigens.

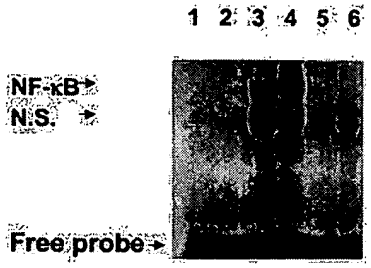


FIG. 1. NF-κB ODN inhibits NF-κB DNA binding in DCs. Nuclear proteins were extracted from DC propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF-κB ODN (NF-κB ODN DC) for 5 days. For further activation, DCs were exposed to LPS (2 μg/ml) for the last 18 h. NF-κB DNA binding activity was determined by ESMA, as described in RESEARCH DESIGN AND METHODS. Medium alone, *lane 1*; DC and cold probe, *lane 2*; DC, *lane 3*; DC stimulated with LPS, *lane 4*; NF-κB ODN DC, *lane 5*; NF-κB DC stimulated with LPS, *lane 6*. NS, nonspecific band. The data are representative of three separated experiments.

RESEARCH DESIGN AND METHODS

Animals. Female NOD ($H2^{g7}$) and C3H ($H2^{k}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center and provided Purina Rodent Chow (Ralston Purina, St. Louis, MO) and water ad libitum. They were used and cared for in accordance with institutional and National Institutes of Health guidelines.

Islet lysate preparation. Islets were isolated from the pancreas by collagenase V (Sigma, St. Louis, MO) digestion as previously described (20), with slight modification (21). After separation on a Ficoll gradient (Type 400; Sigma), the islets were purified by hand picking to eliminate remaining exocrine tissues and suspended in Hank's Balanced Salt Solution (Life Technologies, Grand Island, NY). Islet lysate was prepared by repeat freezing (5 min in dry ice–ethanol bath) and thawing (10 min in 37°C warm bath) four to five times.

ODN. Double-stranded NF-κB ODN decoys were generated using equimolar amounts of single-stranded sense and antisense phosphorothioate-modified ODN containing two NF-κB binding sites (sense strand: 5′-*AGGGACTTTC* CGCTG*GGGACTTTCC*-3′; NF-κB binding sites are italicized) (19). A double-stranded ODN consisting of a random sequence (sense strand: 5′-ACCAGTC CCTAGCTACCAGTCCCTA-3′) was used as control. Sense and antisense strands of each ODN were mixed in the presence of 150 mmol/l NaCl, heated to 100°C, and cooled to room temperature to obtain double-stranded DNA.

DC culture. NOD mouse bone marrow cells were cultured in 24-well plates (2×10^6 per well) in RPMI-1640 media (Life Technologies) supplemented with antibiotics and 10% (vol/vol) FCS (hereafter referred to as "complete medium") containing both granulocyte-macrophage colony-stimulating factor (GM-CSF) (4 ng/ml) and IL-4 (1,000 units/ml) (both from Schering-Plough Research Institute, Kenilworth, NJ) for 5–7 days. The selection and purification procedures were performed as previously described (22). In vitro stimulation of DCs was achieved by exposure to LPS (2 μg/ml) for the last 18 h of culture. To incorporate ODN, 10 μmol/l ODN was added at the initiation of DC culture. For pulsing islet antigens, lysate of pancreatic islets isolated from NOD mice was added at 1:5 of DC:islet cell ratio for the last 48 h of DC culture.

Flow cytometry. Expression of cell surface molecules on DCs was determined by flow cytometric analysis, using an Epics Elite flow cytometer (Coulter Corporation, Hialeah, FL). Cells were stained with the primary hamster or rat monoclonal antibodies (mAbs) against CD40, CD80, CD86, or CD11c followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-hamster IgG or anti-rat IgG_{2a}, as described (5). MHC antigen was detected with FITC-conjugated anti-$H2K^{d}$ mAb (all from B.D. PharMingen, San Diego, CA).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed using a commercially available kit (Promega, Madison, WI) that was supplied with an NF-κB probe oligonucleotide (sense sequence: 5′AGTTGAGGGGACTTTCCCAGGC3′), which was end labeled with γ-^{32}P ATP (NEN, Boston, MA). A 25-fold excess of unlabeled oligonucleotide was used as cold probe. Nuclear proteins (1 μg) were loaded in each lane. The mobility shift was detected by running the mixture on a 4% acrylamide gel. Shifted bands were visualized by autoradiography.

T-cell proliferation. To examine DC allostimulatory activity, γ-irradiated (20 grays) DCs propagated from bone marrow cells of NOD mice were used as stimulators. C3H spleen T-cells (2×10^5) enriched through a nylon wool column were used as responders. For assessment of autostimulatory activity, stimulator DCs were pulsed with islet lysate, and enriched T-cells (2×10^5) isolated from NOD mesenteric lymph nodes or spleens were used as responders. Cells (200 μl/well) were cultured in complete medium in triplicate in 96-well round-bottom plates in 5% CO_2 in air at 37°C for 3–5 days. [^{3}H]TdR (1 μCi/well) was added for the final 18 h of culture. Incorporation of [^{3}H]thymidine into DNA was assessed by liquid scintillation. Results are expressed as mean counts per minute (cpm) ±1 SD.

DC administration and assessment of diabetes. Female 6- to 7-week-old NOD mice were given a single intravenous injection of 2×10^6 DCs, and blood glucose levels were monitored weekly. The first day of two consecutive readings of blood glucose >350 mg/dl was defined as the date of diabetes onset. To score insulitis, pancreata were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 mm each) were prepared and stained with hematoxylin and eosin (23). The lineage of infiltrating cells was identified by immunohistochemistry in cryostat sections using biotinylated rat anti-mouse CD4, CD8, B220, CD11b, or CD11c mAb (B.D. PharMingen) in an avidin-biotin-alkaline phosphase complex staining procedure. Isotype- and species-matched irrelevant mAbs were used as control animals.

Cytokine and nitric oxide quantitation. Levels of IL-2, IFN-γ, IL-4, IL-10, and IL-12 in supernatants of cultures were quantitated using enzyme-linked immunosorbent assay (ELISA) kits (BioSource, Camarillo, CA). Nitric oxide levels were determined by the colorimetric Griess reaction through detecting the end product nitrite (24,25).

RNase protection assay. Total RNA was extracted using the guanidinium isothiocyanate-phenol-chloroform method with TRI reagent (Sigma, St. Louis, MO) as described (24). The purity of RNA was determined from the $A_{260/280}$ absorbance ratio. Cytokine mRNA was assessed using the Ribonuclease Protection Assay Kit (RiboQuant, San Diego, CA). Briefly, probes were synthesized by T7 RNA polymerase with incorporation of α-^{32}P-UTP. Total RNA (5 μg) was treated overnight with synthesized probes (specific activity: 800 Ci · mmol/l) at 56°C, followed by treatment with RNase A (80 μg/ml) and T1 (250 units/ml) for 45 min at 30°C. The murine L32 and glyceraldehyde-3-phosphate dehydrogenase riboprobes were used as controls. Protected fragments were submitted for electrophoresis through a 7.0 mol/l urea/5% polyacrylamide gel and then exposed to Kodak X-omat film for 72 h.

Statistical analysis. Statistical significance was assessed by Student's *t* test or Kaplan-Meier log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

NF-κB ODN inhibits NF-κB DNA binding in DCs. Nuclear proteins extracted from DCs propagated from bone marrow of NOD mice in the absence or presence of NF-κB ODN was assessed for NF-κB DNA binding by EMSA. Specificity of NF-κB binding was demonstrated by utilizing the consensus NF-κB probe and unlabeled NF-κB probe (cold probe) as a competitor. As shown in Fig. 1, the NF-κB band was detected by radiolabeled NF-κB consensus sequence-specific probe in DCs without exposure to NF-κB ODN (*lane 3*). NF-κB binding capacity was en-

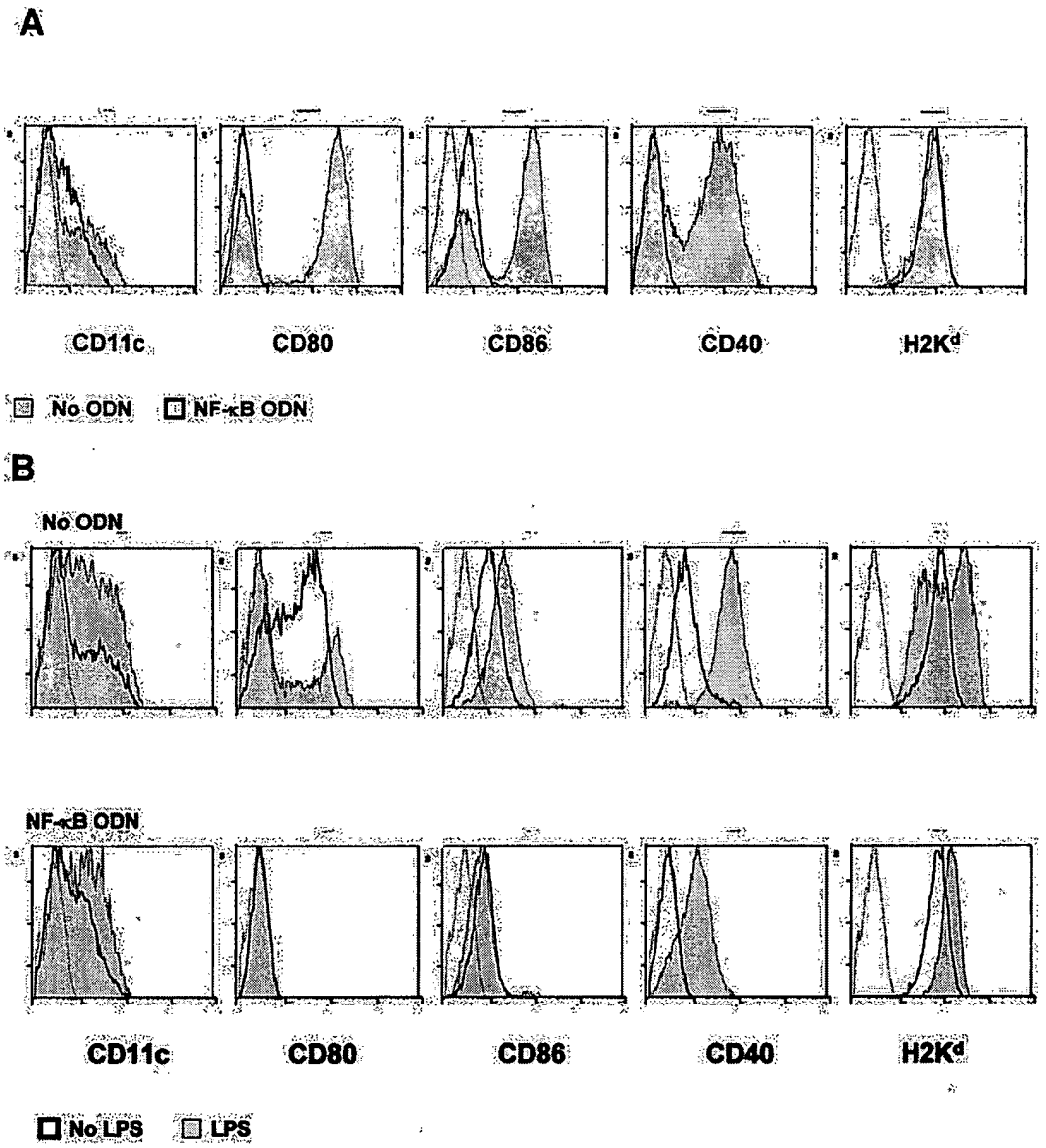


FIG. 2. Inhibition of costimulatory molecule expression on DCs by NF-κB ODN. DCs were propagated from bone marrow of NOD mice in GM-CSF and IL-4 with or without NF-κB ODN (10 μmol/l). For further activation, DCs were exposed to LPS (2 μg/ml) for the last 18 h. Expression of CD40, CD80, CD86, MHC, and CD11c was determined by flow cytometric analysis following mAb staining. Open profiles in dashed lines are isotype controls. *A*: NF-κB ODN markedly suppressed expression of costimulatory molecules, but not MHC and CD11c, on DCs. *B*: LPS significantly augmented CD40, CD80, and CD86 expression in normal DC, while expression of CD40, CD80, and CD86 remained at low levels on NF-κB ODN DC despite LPS stimulation. The data are representative of three separate experiments.

hanced by DC stimulation with LPS (*lane 4*). The NF-κB band disappeared when excess unlabeled probe was added as a competitor for the radiolabeled probe in the binding reaction (*lane 2*). NF-κB ODN almost totally blocked NF-κB binding activity in DCs (*lane 5*). LPS stimulation did not reverse inhibited NF-κB DNA binding capacity by NF-κB ODN (*lane 6*). These data clearly indicate that treatment with NF-κB ODN effectively and stably inhibits NF-κB DNA binding capacity in DCs derived from NOD mice.

NF-κB ODN prevents phenotypical maturation of DCs. Normal DCs propagated from the bone marrow of NOD mice in GM-CSF and IL-4 expressed high levels of MHC and costimulatory molecules, as determined by flow cytometric analysis. NF-κB ODN markedly suppressed surface expression of costimulatory molecules, including CD40, CD80, and CD86 on DCs, but the expression of MHC and DC-restricted marker CD11c was not affected (Fig. 2*A*). MHC and DC-restricted proteins are known to be required for induction of antigen-specific hyporesponsiveness (26). Since LPS-induced DC activation that results in upregulation of CD80, CD86, IL-12, and inducible nitric oxide synthase (iNOS) gene expression is NF-κB dependent (27–29), we examined whether NF-κB ODN decoys prevented LPS-stimulated DC activation. Following exposure to LPS (18 h), there was a significant upregulation in surface expression of CD40, CD80, and CD86 on DCs. Exposure to NF-κB ODN kept CD40, CD80, and CD86 expression at low levels on DCs, even in the presence of LPS stimulation (Fig. 2*B*). This indicates that competitive inhibition of NF-κB DNA binding by NF-κB ODN prevents DC phenotypic maturation, regardless of the presence of

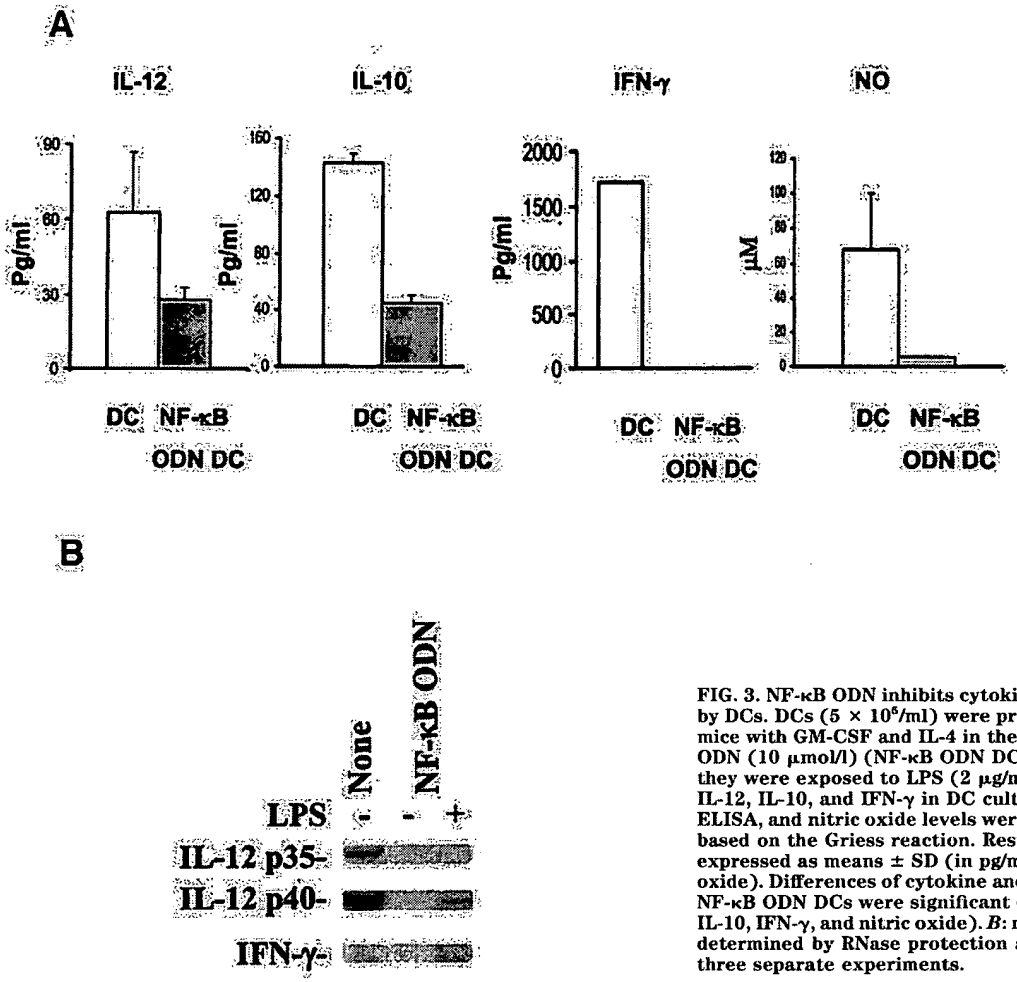


FIG. 3. NF-κB ODN inhibits cytokine and nitric oxide (NO) production by DCs. DCs (5×10^6/ml) were propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF-κB ODN (10 μmol/l) (NF-κB ODN DC) for 5 days. For further activation, they were exposed to LPS (2 μg/ml) for the last 18 h. *A*: The levels of IL-12, IL-10, and IFN-γ in DC culture supernatants were measured by ELISA, and nitric oxide levels were determined by a colorimetric assay based on the Griess reaction. Results from triplicate experiments are expressed as means ± SD (in pg/ml for cytokines and μmol/l for nitric oxide). Differences of cytokine and nitric oxide production by DCs and NF-κB ODN DCs were significant ($P < 0.05$ for IL-12 and $P < 0.01$ for IL-10, IFN-γ, and nitric oxide). *B*: mRNA expression of IL-12. IFN-γ was determined by RNase protection assay. Results are representative of three separate experiments.

the promaturation LPS signal. Therefore, this inhibition is more profound than the inhibition by suppressive cytokines, such as TGF-β, which is easily surmounted by the addition of stimuli, such as allogeneic T-cells or LPS (26).

Effect of NF-κB ODN on DC cytokine profile. NF-κB activation promotes transcription of a number of cytokines, as well as iNOS (27,30). Cytokines produced by DCs provide an important signal for T-cell proliferation, differentiation, and survival. DCs propagated from NOD mice without LPS stimulation produced very low levels of cytokines and nitric oxide (data not shown). LPS stimulation triggered increased DC production of IL-12 (p70), IFN-γ, IL-10, and nitric oxide by DC. In contrast, no IFN-γ and low levels of IL-12 (p70), IL-10, and nitric oxide were detected in the supernatant of NF-κB ODN DCs with LPS stimulation (Fig. 3*A*). Nitric oxide production is NF-κB dependent; therefore, the diminished nitric oxide production in NF-κB ODN DCs was an indication of NF-κB inhibition (29). Consistently, expression of both IL-12 p35, p40, and modest expression of IFN-γ mRNA were shown in non–ODN-treated DCs, but none of these cytokines were detected in NF-κB ODN DCs. LPS stimulation slightly restored expression of IL-12 p40 and IFN-γ in NF-κB ODN DCs, but not IL-12 p35. Expression of IL-1α or -β, the IL-1 receptor, and TNF-α or -β in DCs was not suppressed by treatment with NF-κB ODN, indicating that blocking NF-κB has a minimal effect on the expression of these monokines.

NF-κB ODN inhibits immune stimulatory activity of DCs. The effect of ODN treatment on DC allostimulatory activity was examined in a one-way mixed leukocyte reaction (MLR) assay. Figure 4*A* demonstrates that DCs propagated from NOD mice ($H2^{g7}$) stimulated strong proliferative responses in T-cells isolated from C3H ($H2^k$) spleen. The DC allostimulatory activity was significantly augmented by LPS stimulation. Nevertheless, the T-cell proliferative responses stimulated by NF-κB ODN DCs were markedly suppressed. This inhibition was not reversed by LPS stimulation, indicating the persistent effect of NF-κB ODN. We next assessed the effect of NF-κB ODN on the capacity of DCs to present autoantigens. T-cells from NOD mice were cultured with irradiated NOD DCs pulsed with NOD islet lysate, which provided the relevant autoantigen(s). As shown in Fig. 4*B*, without NF-κB ODN treatment, DCs pulsed with islet antigens effectively stimulated T-cell proliferation, while the autostimulatory activity of NF-κB ODN DCs was significantly suppressed. These data clearly indicate that consistent with the influence of NF-κB ODN on DC surface molecule expression, the in vitro immune stimulatory activity of NF-κB ODN DCs is also significantly suppressed.

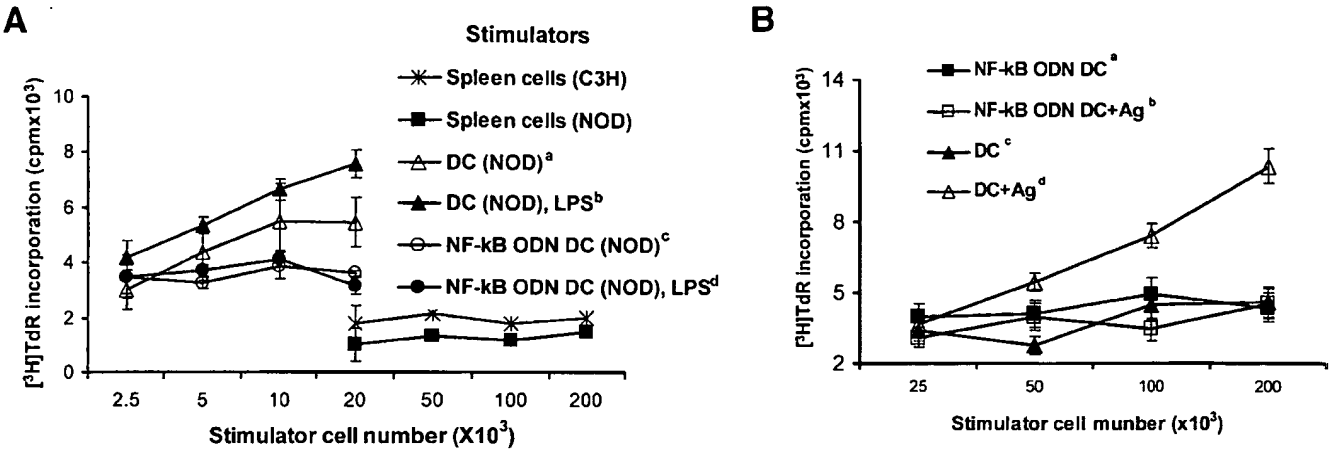


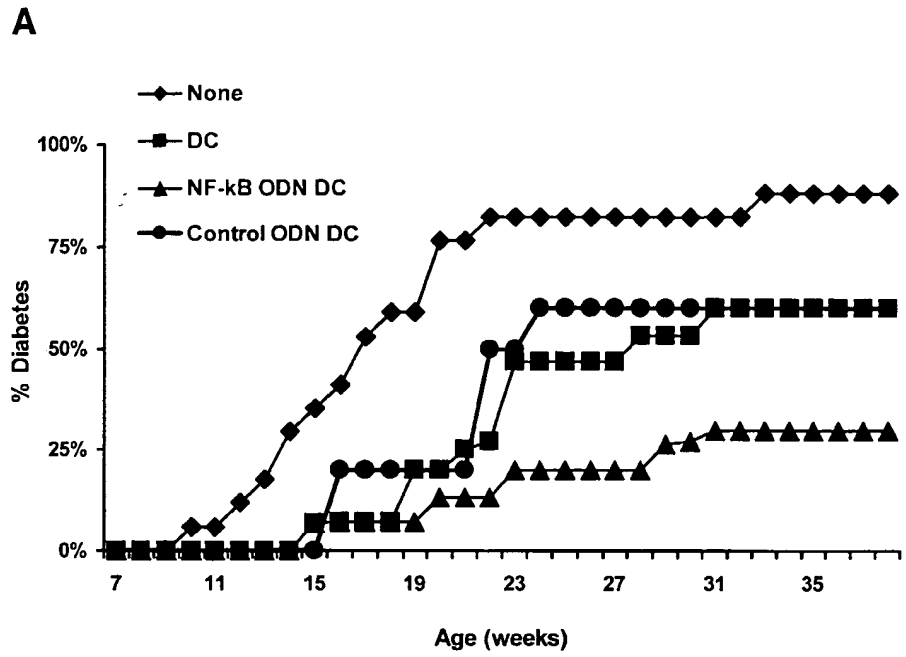
FIG. 4. Inhibition of DC immune stimulatory activity by NF-κB ODN. DCs were propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF-κB ODN (10 μmol/l) (NF-κB ODN DC). For further activation, DCs were exposed to LPS (2 μg/ml) for the last 18 h. *A*: C3H ($H2^k$) splenic T-cells (2×10^5) were cultured with γ-irradiated DCs propagated from NOD mice ($H2^{g7}$) at graded concentrations for 3 days. Incorporation of [^{3}H]TdR into DNA demonstrated that LPS stimulation augmented DC allostimulatory function ($P < 0.05$, a vs. b at 2×10^4 stimulators), while NF-κB ODN inhibited DC allostimulatory activity ($P < 0.05$, a vs. c at 2×10^4 stimulators; $P < 0.01$, b vs. d at both 2×10^4 and 1×10^4 stimulators), which was irreversible by LPS ($P > 0.05$, c vs. d at all stimulator:responder ratios). Spleen cells from C3H and NOD mice were used as control stimulators. *B*: Spleen T-cells (2×10^5) of NOD mice were cultured for 5 days with γ-irradiated DCs propagated from NOD mice pulsed with or without islet lysate from NOD mice. DCs pulsed with islet antigens induced substantial T-cell proliferative responses ($P < 0.01$, c vs. d at both 1×10^3 and 2×10^5 stimulators), which was significantly reduced by treatment of DCs with NF-κB ODN ($P < 0.01$, b vs. d at both 1×10^3 and 2×10^5 stimulators). The results are expressed as counts per minute (cpm) (means ± SD) and are representative of three separate experiments.

Administration of NF-κB ODN DCs inhibits development of diabetes. Our net effort was directed toward assessing preventive effects of administration of NF-κB ODN DCs on type 1 diabetes. Female NOD mice spontaneously developed pathological insulitis, which was evident at 6–8 weeks of age with peri-islet infiltration followed by a progressive destruction of pancreatic β-cells. Histological examination revealed that the insulitis score increased from 2.18 ± 1.25 at 12 weeks of age to 4.0 ± 0 at 20 weeks of age, with 88% of the mice developing destructive insulitis and diabetes by the age of 20 weeks (Fig. 5*A* and *C*). The early inflammatory changes as a result of peri-insulitis were characterized by infiltration of macrophages ($CD11b^+$) and DCs ($CD11c^+$). After 12 weeks of age, $CD4^+$ T-cells were the predominant population of the infiltrates, accompanied by increased numbers of macrophages and DCs mixed with $CD8^+$ T-cells (Fig. 5*D*). NOD mice were treated at 6–7 weeks of age with a single intravenous injection of 2×10^6 NF-κB ODN DCs propagated from bone marrow from NOD mice. Three control groups of age-matched NOD mice included administration of normal DCs, control ODN DCs, and none. A single intravenous administration of NF-κB ODN DCs protected 70% of NOD mice from the development of diabetes up to the age of 38 weeks (Fig. 5*A*). Injection of normal DCs could protect ~30% of the mice from developing of diabetes by the age of 38 weeks, instead of accelerating the progression of disease. Similar findings reported by other investigators (31) show that normal mature DCs propagated from NOD bone marrow have a slight effect on diabetes protection in NOD mice. The diabetes incidence in the control ODN-DC treatment group is similar to that in the normal DC treatment group (Fig. 5*A*). The insulitis and islet infiltrates were histologically examined. As demonstrated in Fig. 5*B*, the severity of histological insulitis was markedly diminished with substantial reduction in peri- and intra-islet infiltration at 30 weeks of age in mice treated with NF-κB ODN DCs compared with that of non–DC-treated mice at as early as 12 weeks of age. The pancreata from three mice in each group were scored for insulitis as described in RESEARCH DESIGN AND METHODS. The insulitis score in the NF-κB ODN DC group was significantly lower than that in the non-DC and normal DC treatment groups (Fig. 5*C*). Compared with the non-DC treatment group, $CD11b^+$ macrophages and $CD8^+$ T-cells were significantly reduced in the subcapsular areas in NF-κB ODN DC–treated mice, while $CD4^+$ T-cells were markedly increased (Fig. 5*D*). Administration of NF-κB ODN DC pulsed in vitro with islet lysates did not further improve the ability of these cells to prevent disease (data not shown). This may implicate that process of in vivo existing antigens by adoptively transferred NF-κB ODN DCs is sufficient to prevent diabetes.

TABLE 1
IL-2 and IFN-γ levels in supernatants from MLR cultures.

Treatment	Ag^-		Ag^+	
	IL-2	IFN-γ	IL-2	IFN-γ
None	22.3 ± 7.6	ND	5.5 ± 7.8	9.2 ± 0.2
DC	103.8 ± 4.7	520.5 ± 28.2	97.7 ± 4.7	4,000 ± 0
NF-κB DC	0.16 ± 0.22*	ND†	ND‡	ND§

Data are mean cpm ± 1 SD and are representative of three separate experiments. Responders: T-cells (2×10^5) purified from mesenteric lymph nodes of NOD mice 4 weeks after intravenous administration of DCs (2×10^6) that were propagated from bone marrow of NOD mice in the absence or presence of NF-κB ODN (10 μmol/l). T-cells from NOD mice without DC treatment were used as controls. Stimulators: γ-irradiated DCs propagated from NOD mice bone marrow that were pulsed with islet antigens from NOD mice in vitro (Ag^+) or not pulsed with antigens (Ag^-). Responders were cultured for 5 days with stimulator at an stimulator:responder ratio of 1:10. Cytokine levels in the culture supernatants were determined by ELISA. *$P < 0.05$ vs. none, $P < 0.01$ vs. DCs; †$P > 0.05$ vs. none, $P < 0.01$ vs. DCs; ‡$P > 0.05$ vs. none, $P < 0.01$ vs. DCs; §$P < 0.01$ vs. none, $P < 0.01$ vs. DCs. ND, not detectable.



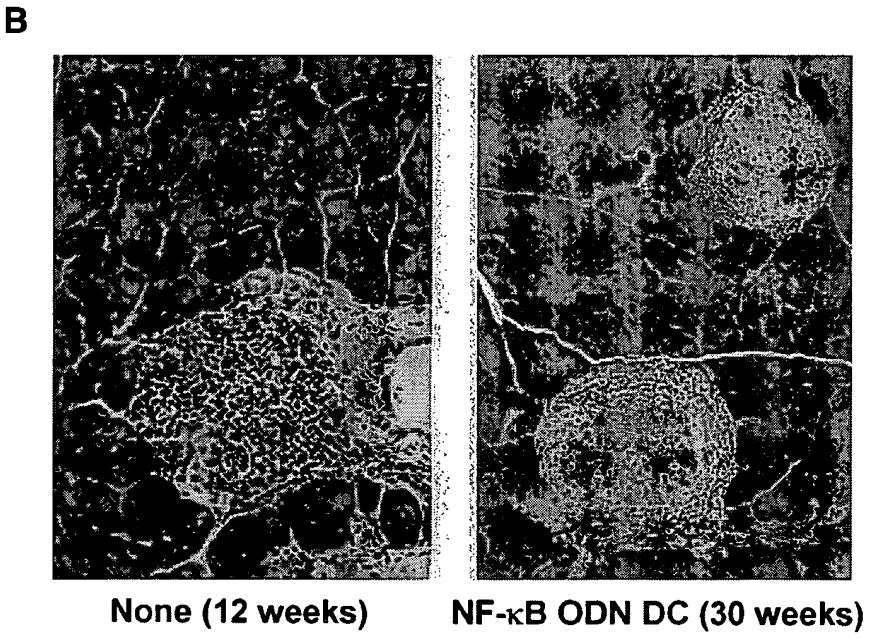


FIG. 5. Systemic administration of NF-κB ODN DCs prevents diabetes development in NOD mice. NOD mice were injected intravenously at 6–7 weeks of age with 2×10^6 DCs propagated from NOD bone marrow with GM-CSF and IL-4 in the presence of NF-κB ODN (NF-κB ODN DC, $n = 17$) or control ODN (control ODN DC, $n = 8$) or in the absence of ODN (DC, $n = 15$). Mice without DC treatment (none, $n = 15$) were also used as control subjects. *A*: Without DC treatment, 88% of the mice developed diabetes at the age of 33 weeks. The diabetes incidence in the control ODN-DC treatment group is similar to the non–ODN-treated DC group, showing modestly prevented diabetes development. Administration of NF-κB ODN DCs markedly reduced development of diabetes ($P < 0.01$, compared with either non–ODN DC or control ODN DC group). *B*: Histology of pancreatic islets (hematoxylin and eosin) from NOD mice treated with NF-κB ODN DC at 30 weeks of age revealed very minimal insulitis, and untreated NOD mice developed severe insulitis as early as 12 weeks of age (magnification ×400). *C*: Three mice in each group were scored (50 islets per mouse) for insulitis, as described in RESEARCH DESIGN AND METHODS, indicating that insulitis was significantly less severe in mice treated with NF-κB ODN DCs than those treated with normal DCs or without DC treatment (both $P < 0.05$). *D*: Cryostat sections of pancreata were stained with anti-CD4, -CD8, -CD11c, or -CD11b mAbs (magnification ×200). The pictures are representative of three separated experiments. For quantitation, 30 high-power fields (hpf) of islet subcapsular areas in each mouse were randomly selected and counted for staining positive cells. The data were collected from three mice in each group and are presented as positive cell numbers/hpf (±1 SD).

Administration of NF-κB ODN DCs induces T-cell hyporesponsiveness to islet antigens. To examine the effect of NF-κB ODN DC administration on T-cell responses in vivo, lymphocytes isolated from NOD spleen or mesenteric lymph nodes 4 weeks after DC treatment were assessed for T-cell proliferative responses to islet lysate in MLR where NOD DCs pulsed with pancreatic islet lysate were stimulators. T-cells from mice treated with normal DCs developed a strong proliferative response to islet antigens (Fig. 6), which was associated with increased production of IFN-γ and IL-2 (Table 1), reflecting a bias toward a T-helper 1 response. In contrast, T-cells from the NF-κB ODN DC–treated group exhibited low proliferation, along with decreased production of IFN-γ and IL-2 on in vitro restimulation (Fig. 6 and Table 1). Low levels of IL-4 and IL-10 were detectable in all groups of mice (data not shown). These results suggest that NF-κB ODN DCs elicit hyporesponsiveness of autoreactive T-cells in NOD mice, partly by suppression of T-helper 1 responses.

DISCUSSION

We demonstrate in this study that administration of syngeneic bone marrow–derived DCs, in which NF-κB activity is blocked by a designed decoy ODN, effectively prevents development of diabetes in NOD mice. At 38 weeks of age, 70% of the mice were rendered normoglycemic by one injection of NF-κB ODN DCs. This was associated with markedly inhibited autoreactive T-cell responses and T-helper 1 responses (Fig. 3). It is unlikely

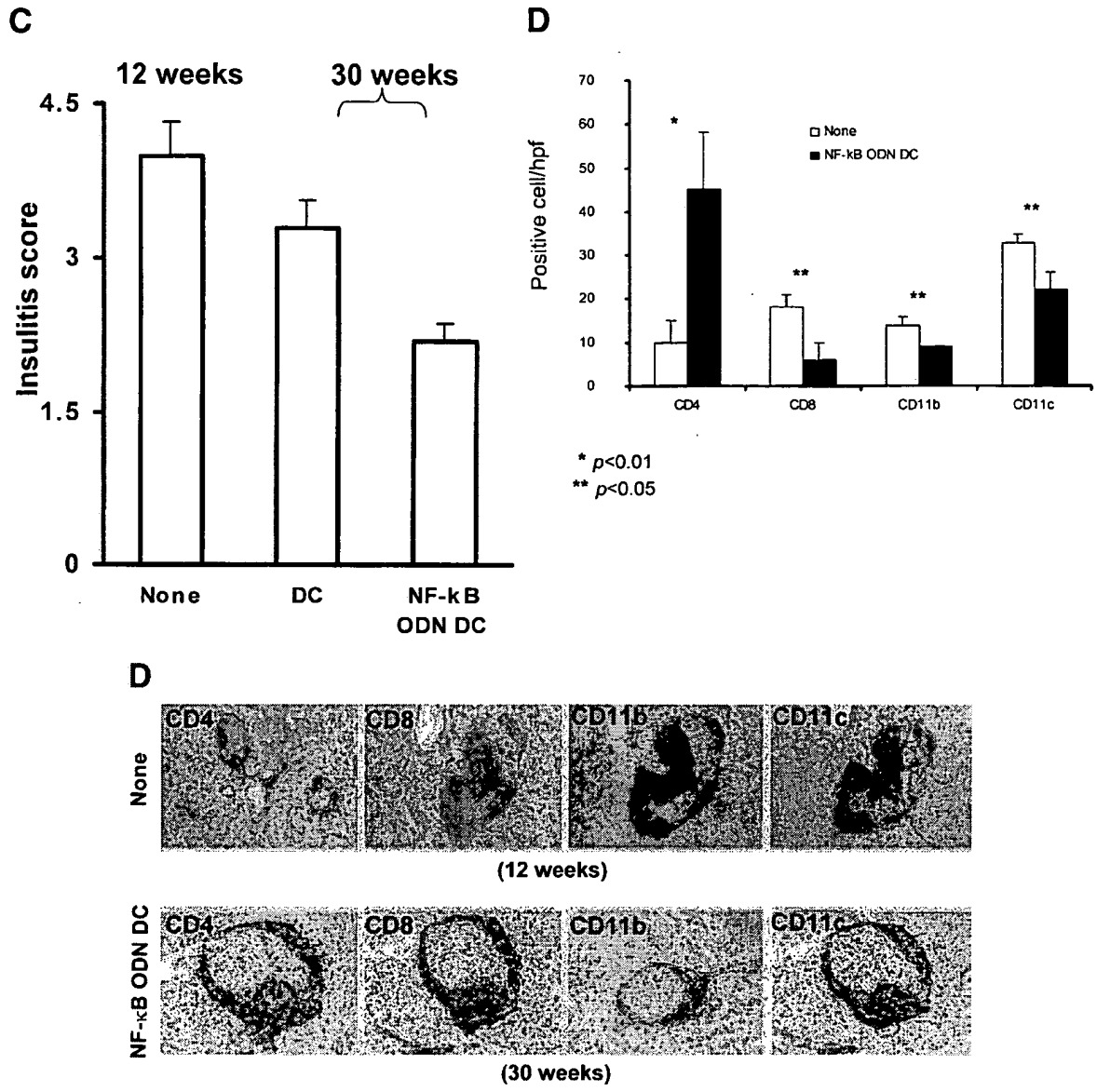


FIG. 5. Continued

that the suppressed T-cell responses by NF-κB ODN DCs are a consequence of ODN toxicity. We have previously determined the "safety window" for ODN incorporation in DCs by determining the viability of DC using dye exclusion, as well as quantitative measurement of apoptosis with transferase-mediated dUTP nick-end labeling

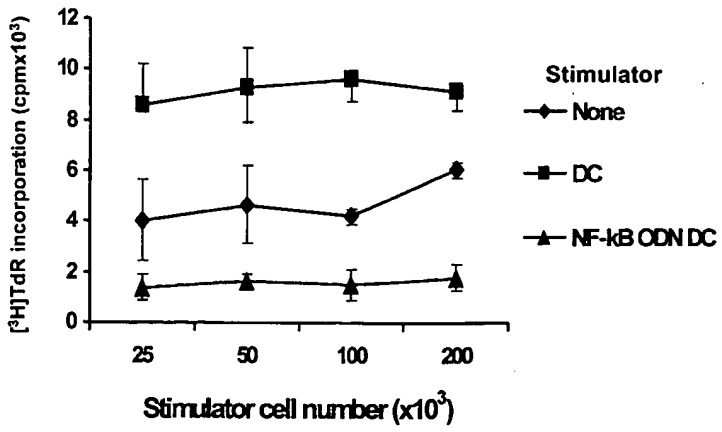


FIG. 6. Administration of NF-κB ODN DCs to NOD mice induces T-cell hyporesponsiveness to islet antigens. In a 4-day MLR assay, T-cells (2×10^3) purified from mesenteric lymph nodes of NOD mice that had received, at 6–7 weeks of age, 2×10^6 DCs intravenously were used as responders. γ-Irradiated DCs that were propagated from bone marrow of NOD mice with GM-CSF and IL-4 pulsed with islet lysates were used as stimulators. The results are expressed as counts per minute (cpm) (means ± 1 SD) and are representative of three separate experiments.

(TUNEL) staining. Using FITC-conjugated NF-κB ODN, we observed that DCs effectively incorporated ODN and survived for as long as untreated DCs. DCs treated with control ODN stimulated a strong allogeneic MLR (19). We did not observe a reduction in DC number following NF-κB ODN treatment at 10 nmol/l in the present study. These results indicate that exposure of DCs to 10 nmol/l NF-κB ODN yielded a satisfactory suppressive effect with minimal toxicity.

The results of this study coincide with the view that type 1 diabetes is initiated by autoreactive T-cell responses toward self-antigens. Indeed, T-cells from the NOD mice displayed abnormally high reactivity to self-proteins (32) and T-helper 1 responses to the autoantigens (33,34). Activation of APCs, including DCs, and subsequent migration from nonlymphoid tissues to regional lymph nodes are primary events elicited during inflammatory processes and are critical for the generation of cellular responses against self-antigens in type 1 diabetes (35). This was first suggested by the fact that adoptive transfer of DCs pulsed with a self-antigen expressed in islet β-cells, using transgene technology, activated the antigen-specific cytotoxic T-lymphocytes (CTLs) and promoted development of autoimmune diabetes, indicating that autoreactive CTL stimulation by enhanced DC antigen-presentation function is an important prerequisite for the progression of diabetes (36). DCs are also an important source of TNF-α in islet infiltrates during the early developmental stages of diabetes, which may contribute to β-cell destruction (10). The functional defects of DCs in NOD mice were correlated with significantly enhanced activity of NF-κB (11,18). Signaling by members of the TNF-α receptor family, such as CD40 and receptor activator of NF-κB (RANK), or LPS results in activation of NF-κB in DCs, leading to upregulation of costimulatory molecule expression and enhancement of IL-12 production (11). The association of immunogenic DC activity and β-cell destruction in NOD mice suggests that certain β-cell antigens are processed by the immunogenic DCs, resulting in the delivery of a signal that stimulates the autoimmune responses that lead to islet cell destruction. Therefore, it has been recently proposed (21) that an imbalance favoring development of the immunogenic DCs may predispose NOD mice to the development of autoimmune diabetes. The prevention of diabetes development in NOD mice by administration of NF-κB ODN–treated DCs at a prediabetic stage (6 weeks), as shown in this study, may reflect a consequence of the restoration of the balance between immunogenic and tolerogenic DC function, in which hyporesponsiveness of antigen-specific T-cells via the inhibition of T-helper 1 differentiation results. These results are consistent with the reports of other investigators in which immature DCs used as therapeutic vectors or adjuvants diminished or regulated autoimmune responses in type 1 diabetes (31).

Although the effect of immature DC administration on regulating immune responses has previously been demonstrated in other experimental models (31), approaches to generating DCs in a stable immature state remain to be developed. Immature DCs were initially obtained for inhibition of maturation in culture by using inhibitory cytokines, such as transforming growth factor-β. In an allograft transplantation model, administration of these cytokine-induced immature DCs induced donor-specific hyporesponsiveness and prolonged allograft survival (37). However, the immunosuppressive effect of these immature DCs is limited due to late activation following in vivo contact with inflammatory cytokines or activated T-cells (26). Genetically engineering DCs to express "designer" immunosuppressive molecules, such as IL-10, TGF-β, CTLA4Ig, or Fas ligand, in order to enhance tolerogenicity, has been explored. Adenoviral vectors can efficiently deliver the transgenes into DCs, but transgene expression is surmounted by the vigorous upregulation of costimulatory molecules on DCs by stimulation of the viral vectors themselves (38,39). Blockade of NF-κB binding to inhibit DC maturation is a new approach that is attracting extensive attention. NF-κB activation can be inhibited at each step by pharmacological agents, including glucocorticoid-dependent upregulation of IκB, Cyclosporine A–dependent calcineurin inhibition, and deoxyspergualin-dependent inhibition of nuclear translocation (40). The therapeutic use of these agents is, however, limited by weak NF-κB inhibition. The NF-κB ODN decoys used in this study effectively inhibit both phenotypic and functional maturation in DCs. NF-κB ODN not only inhibits, but also resists, the maturation of DCs in response to LPS stimulation, thus indicating a stable immature state. Moreover, NF-κB ODN selectively suppresses expression of costimulatory molecules without affecting the DC-restricted marker CD11c and MHC expression, both of which are known to be required for DCs in the induction of antigen-specific tolerance (26). Suppression of IL-12 production by NF-κB ODN DCs may contribute to the therapeutic efficacy of these modified DCs because the islet-infiltrating T-cells in NOD mice expressed high T-helper 1 (IFN-γ and IL-2) and T-helper 1–related cytokines (IL-12) (23,33,41,42) and treatment with anti–IL-12 mAb suppresses T-helper 1–dependent islet destruction (43).

The reduction in intra-islet infiltration of macrophages, DCs, and $CD8^+$ T-cells in NOD mice treated with NF-κB ODN DCs suggests that the inhibition may involve both afferent and efferent arms of the immune system. It is unlikely that the decreases in effector $CD8^+$ infiltrates are due to a lack of immune stimulation, since significant $CD4^+$ infiltrates were observed following treatment with NF-κB ODN DCs. The enhanced $CD4^+$ T-cell infiltration by NF-κB ODN DC treatment was unexpected, as earlier results demonstrated that $CD4^+$ T-cells contributed to insulitis development and transfer of $CD4^+$ islet-specific T-cells into NOD mice accelerated diabetes onset (44). The difficulty in isolating sufficient cells from islets prevented us from further characterizing the $CD4^+$ infiltrates. However, T-cells from the lymph nodes of NF-κB ODN DC–treated NOD mice secreted low IFN-γ and IL-2 when restimulated in vitro by islet antigens in vitro, suggesting the downregulation of T-helper 1 differentiation. Immature DCs have also been reported (6) to promote differentiation of T regulatory cells. The $CD4^+$ T-cells in the islets of nondiabetic mice treated with NF-κB ODN DCs may implicate the presence of a regulatory population with immunosuppressive properties (45–49).

Interestingly, despite the untreated mature phenotype and stimulatory function of mature DC propagated from NOD bone marrow in the presence of GM-CSF and IL-4 in

vitro, a single injection of them modestly reduced the incidence of diabetes in NOD mice (Fig. 5) rather than accelerated disease development. Other investigators have reported similar observations (31). These data are in contrast to those observed in our allograft transplant model, in which the administration of mature donor-derived DC exacerbates graft rejection (37). The disparate outcome may result from different antigens and the way the antigens are being presented. In NOD mice, the administered mature DCs process and indirectly present autoantigens to T-cells in vivo, thereby leading to a regulatory T-helper 2 response (50). Another possibility is that in NOD mice, the mature DCs may act like BCG or complete Freund's adjuvant to nonspecifically inhibit T-cell responses to islet antigens since the DC culture medium contains antigens, such as fetal bovine serum.

In summary, our data suggest that NF-κB ODN effectively inhibits the maturation/activation of DCs propagated from NOD mice. Compared with mature DCs, administration of DCs with suppressed NF-κB DNA binding activity can more effectively prevent diabetes development and induce diabetogenic T-cell hyporesponsiveness in NOD mice. This strategy may be utilized for selective prevention or therapy for human autoimmune diseases, including type 1 diabetes.

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REFERENCES

1. Bach JF: Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr Rev* 15:516–542, 1994
2. Ludewig B, Odermatt B, Ochsenbein AF, Zinkernagel RM, Hengartner H: Role of dendritic cells in the induction and maintenance of autoimmune diseases. *Immunol Rev* 169:45–54, 1999
3. Lechler R, Ng WF, Steinman RM: Dendritic cell in transplantation: friend or foe? *Immunity* 14:357–368, 2001
4. Banchereau J, Briere F, Caux C, Davoust S, Lebecque S, Liu JY, PulendranB, Palika K: Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767–811, 2000
5. Lu L, Mccaslin D, Starzl TE, Thomson AW: Bone marrow-derived dendritic cell progenitors (NLDC 145^+, MHC classII$^+$, B7-1^{dim}, B7-2^-) induce alloantigen-specific hyporesponsiveness in murine T lymphocytes. *Transplantation* 60:1539–1545, 1995
6. Roncarolo M-G, Levings MK, Traversari C: Differentiation of T regulatory cells by immature dendritic cells (Review). *J Exp Med* 193:F5–F9, 2001
7. Takahashi K, Honeyman MC, Harrison LC: Impaired yield, phenotype, and function of monocyte-derived dendritic cells in humans at risk for insulin-dependent diabetes. *J Immunol* 161:2629–2635, 1998
8. Jansen A, van Hagen M, Drexhage HA: Defective maturation and function of antigen-presenting cells in type I diabetes. *Lancer* 345:491–492, 1995
9. Serreze DV, Gaskins HR, Leiter EH: Defects in the differentiation and function of APCs in NOD/Lt mice. *J Immunol* 150:2534–2543, 1993
10. Green EA, Flavell RA: Tumor necrosis factors and the progression of diabetes in non-obese diabetic mice. *Immunol Rev* 167:11–22, 1999
11. Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P: Dendritic cell survival and maturation are regulated by different signaling pathways. *J Exp Med* 188:2175–2180, 1998
12. Weih F, Carrasco D, Durham SK, Barton DS, Rizzo CA, Ryseck RP, Lira SA, Bravo R: Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family. *Cell* 80:331–340, 1995
13. Wu L, D'Amico A, Winkel K, Suter M, Lo, Shortman K: RelB is essential for the development of myeloid-related CD8α^+ dendritic cells but not of lymphoid-related CD8α^+ dendritic cells. *Immunity* 9:839–847, 1998
14. Thanos D, Maniatis T: NF-κB: a lesson in family values. *Cell* 80:592–532, 1995
15. May MJ, Ghosh S: Signal transduction through NF-κB. *Immunol Today* 19:80–88, 1998
16. Weaver DJ Jr, Poligone B, Bui T, Abdel-Motal UM, Baldwin AS Jr, Tisch R: Dendritic cells from nonobese diabetic mice exhibit a defect in NF-κB regulation due to a hyperactive IκB kinase. *J Immunol* 167:1461–1468, 2001
17. Poligone B, Weaver DJ Jr, Sen P, Baldwin AS Jr, Tisch R: Elevated NF-κB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J Immunol* 168:188–196, 2002
18. Steptoe RJ, Ritchie JM, Harrison LC: Increased generation of dendritic cells from myeloid progenitors in autoimmune-prone nonobese diabetic mice. *J Immunol* 168:5032–5041, 2002
19. Giannoukakis N, Bonham CA, Qian, Chen Z, Peng L, Harnaha J, Li W, Thomson AW, Fung JJ, Robbins PD, Lu L: Prolongation of cardiac allograft survival using dendritic cells treated with NF-κB decoy oligodeoxyribonucleotides. *Mol Ther* 1:430–437, 2000
20. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP: An improved method for isolation of mouse pancreatic islets. *Transplantation* 40:437–438, 1985
21. Alexander AM, Crawford M, Bertera S, Rudert WA, Takikawa O, Robbins PD, Trucco M: Indoleamine 2,3-dioxygenase expression in transplanted NOD islets prolongs graft survival after adoptive transfer of diabetogenic splenocytes. *Diabetes* 51:356–365. 2002
22. Lu L, Qian S, Hershberger PA, Rudert WA, Li Y, Chambers FG, Lynch DH, Thomson AW: Fas ligand (CD95L) and B7 expression on dendritic cells provide counter-regulatory signals for T-cell survival and proliferation. *J Immunol* 158:5676–5684, 1997
23. Kim KS, Kang Y, Choi SE, Kim JH, Kim HM, Sun B, Jun HS, Yoon JW: Modulation of glucocorticoid-induced GAD expression in pancreatic β-cells by transcriptional activation of the GAD67 promoter and its possible effect on the development of diabetes. *Diabetes* 51:2764–2772, 2002
24. Lu L, Bonham CA, Liang X, Zhen Z, Li W, Wang L, Watkins S, Nalesnik MA, Schlissel MS, Demestris AJ, Fung JJ, Qian S: Liver-derived DEC205^+ B220^+ CD19^- dendritic cells regulate T-cell response. *J Immunol* 166:7042–7052, 2001
25. Lu L, Bonham CA, Chambers FG, Watkins SC, Hoffman RA, Simmons RL, Thomson AW: Induction of nitric oxide synthase in mouse dendritic cells by IFN-γ, endotoxin, and interaction with allogeneic T-cells: nitric oxide production is associated with dendritic cell apoptosis. *J Immunol* 157: 3577–3586, 1996
26. Lu L, Li W, Zhong C, Qian S, Fung JJ, Thomson AW, Starzl TE: Increased apoptosis of immunoreactive host cells and augmented donor leukocyte chimerism, not sustained inhibition of B7 molecule expression are associated with prolonged cardiac allograft survival in mice preconditioned with immature donor dendritic cells plus anti-CD40L mAb. *Transplantation* 68:747–757, 1999
27. Lu L, Thomson AW: Dendritic cells tolerogenicity and prospects for dendritic cell-based therapy of allograft rejection and autoimmune disease. In *Dendritic Cells: Biology and Clinical Applications*. 2nd ed. Lotze MT, Thomson AW, Eds. London, Academic Press, 2001, p. 587–607
28. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM: Regulation of interleukin-12 p40 expression through an NF-κB half-site. *Mol Cell Bio* 5:5258–5267, 1995
29. Xie QW, Kashiwabara Y, Nathan C: Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J Biol Chem* 269:4705–4708, 1994
30. Rissoan, M-C, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, Liu Y-J: Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283:1183–1186, 1998
31. Feili-Hariri M, Dong X, Alber SM, Watkins SC, Salter RD, Morel PA: Immunotherapy of NOD mice with bone marrow-derived dendritic cells. *Diabetes* 48:2300–2308, 1999
32. Ridgway WM, Fasso J, Lanctot A, Garvey C, Fathman CG: Breaking self-tolerance in nonobese diabetic mice. *J Exp Med* 183:1657–1662, 1996
33. Trembleau 5, Penna G, Bosi B, Mortara A, Gately MK, Adorini L: Interleukin 12 administration induces T helper type 1 cells and accelerates autoinumune diabetes. *J Exp Med* 181:817–821,1995
34. Healey D, Ozegbe P, Arden 8, Chandler P, Hutton J, Cooke A: In vivo activity and in vitro specificity of CD4^+ Th1 and Th2 cells derived from the spleens of diabetic NOD mice. *J Clin Invest* 95:2979–2985, 1995
35. Bach JF, Chatenoud L: Tolerance to islet autoantigens in type 1 diabetes. *Ann Rev Immunol* 19:131–161, 2001
36. Ludwig B, Odermatt B, Landmann S, Hengartner H, Zinkenagel: Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue. *J Exp Med* 188:1493–1501, 1998

37. Fu F, Li Y, Qian S, Lu L, Chambers FD, Starzl TE, Fung JJ, Thomson AW: Costimulatory molecule-deficient dendritic cell progenitors (MHC class II^+, B7-1^{dim}, B7-2^-) prolong cardiac allograft survival in non-immunosuppressed recipients. *Transplantation* 62:659–665, 1996
38. Lu L, Lee WC, Gambotto A, Zhong C, Robbin PD, Qian S, Fung JJ, Thomson AW: Transduction of dendritic cells with adenoviral vectors encoding CTLA4-Ig markedly reduces their allostimulatory activity. *Transplant Proc* 31:797, 1999
39. Lee WC, Zhong C, Qian S, Wan Y, Gauldie J, Mi Z, Robbins PD, Thomson AW, Lu L: Phenotype, function, and in vivo migration and survival of allogeneic dendritic cell progenitors genetically engineered to express TGF-β. *Transplantation* 66:1810–1817, 1998
40. Lee J, Burckart GJ: Nuclear factor kappa B: important transcription factor and therapeutic target. *J Clin Pharmacol* 38:981–993, 1998
41. Chung Y-H, Jun H-S, Kang Y, Hirasawa K, Lee B-R, Rooijien NV, Yoon J-W: Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced autoimmune diabetes in diabetes-resistant BioBreeding rats. *J Immunol* 159:466–471, 1997
42. Alleva DG, Pavlovich RP, Grant C, Kaser SB, Beller DI: Aberrant macrophage cytokine production is a conserved feature among autoimmune-prone mouse strains: elevated interleukin (IL)-12 and an imbalance in tumor necrosis factor-α and IL-10 define a unique cytokine profile in macrophages from young nonobese diabetic mice. *Diabetes* 49:1106–1115, 2000
43. Hayashi T, Morimoto M, Iwata H, Onodera T: Possible involvement of IL-12 in reovirus type-2-induced diabetes in newborn DBA/1 mice. *Scandinavian J Immunol* 53:572–578, 2001
44. Haskins K, McDuitie M: Acceleration of diabetes in young NOD mice with a $CD4^+$ islet-specific T-cell clone. *Science* 249:1433–1436, 1990
45. Shevach EM: Regulatory T-cells in autoimmunity. *Ann Rev Immunol* 18:423–449, 2000
46. Takahashi T, Tagami T, Yamazaki S, Uede I, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S: Immunologic self-tolerance maintained by $CD25^+$ $CD4^+$ regulatory T-cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192:303–310, 2000
47. Read S, Malmstroni V, Powrie F: Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+$ $CD4^+$ regulatory cells that control intestinal inflammation. *J Exp Med* 192:295–302, 2000
48. Groux H, O'Garra A, Bigler M, Rouleau M, Antinenko S, de Vries JE, Roncarolo M-G: A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737–742, 1997
49. Roncarolo M-G, Levings MK: The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr Opin Immunol* 12:676–683, 2000
50. Feili-Hariri M, Falkner DH, Morel PA: Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice. *European J Immunol* 32:2021–2030, 2002

Brief Definitive Report

Interleukin 12 Administration Induces T Helper Type 1 Cells and Accelerates Autoimmune Diabetes in NOD Mice

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Summary

T cells play a major role in the development of insulin-dependent diabetes mellitus (IDDM) in nonobese diabetic (NOD) mice. Administration of interleukin 12 (IL-12), a key cytokine which guides the development of T helper type 1 (Th1) $CD4^+$ T cells, induces rapid onset of IDDM in NOD, but not in BALB/c mice. Histologically, IL-12 administration induces massive infiltration of lymphoid cells, mostly T cells, in the pancreatic islets of NOD mice. $CD4^+$ pancreas-infiltrating T cells, after activation by insolubilized anti T cell receptor antibody, secrete high levels of interferon γ and low levels of IL-4. Therefore, IL-12 administration accelerates IDDM development in genetically susceptible NOD mice, and this correlates with increased Th1 cytokine production by islet-infiltrating cells. These results hold implications for the pathogenesis, and possibly for the therapy of IDDM and of other Th1 cell–mediated autoimmune diseases.

Based on the repertoire of lymphokine production, mouse (1) and human (2) $CD4^+$ T cells can be subdivided into two subsets, Th1 and Th2, characterized by secretion of IFN-γ and IL-4, respectively. The generation of Th1 and Th2 subsets is influenced by the cytokines present during the initial phase of the immune response, and a major role is played by IL-12 and IL-4 (3, 4). IL-12, a heterodimeric cytokine (5, 6) produced by activated monocytes and B cells (7), promotes Th1 cell development. It also enhances proliferation and cytolytic activity of NK and T cells (8), and stimulates production of IFN-γ by these cell types (9). IFN-γ appears to play a role in the development of insulin-dependent diabetes mellitus (IDDM), as demonstrated by the prevention of disease by administration of anti-IFN-γ mAb to nonobese diabetic (NOD) mice (10, 11), or by IDDM induction in mice expressing genes encoding IFN-γ under the control of the insulin promoter (12). It has been proposed that the pathological immune response of NOD mice to islet β cells is initiated by $CD4^+$ T cells that recognize glutamic acid decarboxylase (13, 14), and splenic $CD4^+$ T cells from NOD mice secrete IFN-γ when stimulated with this enzyme (13). Conversely, the Th2-derived lymphokines IL-4 and IL-10 appear to inhibit progression to IDDM in NOD mice (15–17). These findings suggest that T cells that recognize pancreatic β cell antigens cause IDDM only if they develop into Th1 cells.

To test this assumption, we treated prediabetic NOD mice with IL-12, which has been shown to induce the development of IFN-γ–producing Th1 cells in vitro (18, 19) and in vivo (20). Results in the present paper demonstrate that administration of IL-12 to prediabetic NOD female mice induces rapid onset of IDDM. This is associated with enhanced production of Th1-type cytokines by islet-infiltrating lymphocytes, and with selective destruction of islet β cells.

Materials and Methods

Mice. 10-wk-old female BALB/c mice were obtained from Charles River (Calco, Italy). NOD/Lt mice from The Jackson Laboratory (Bar Harbor, ME) were bred and kept in conventional housing conditions in our animal facility. Mice were diagnosed diabetic after two sequential measurements of blood glucose levels >200 mg/dl. The incidence of IDDM in NOD female mice from our colony was ~60%.

Recombinant Mouse IL-12. Recombinant mouse IL-12 was produced in serum-free medium by transfected CHO cells and purified by sequential chromatography, as described (21). The IL-12 used in this study was >95% pure, as assessed by SDS-PAGE analysis, and the endotoxin content was <5 endotoxin U/mg IL-12, as determined by the *Limulus* amebocyte assay. IL-12 was diluted in PBS containing 1% syngenic mouse serum or 100 μg/ml mouse serum albumin (Sigma Chemical Co., St. Louis, MO) and injected intraperitoneally in a 0.2-ml volume. IL-12 has a half-life in mice of ~5 h (Gately, M., and R. Nadeau, unpublished observations).

Isolation of Pancreas-infiltrating Cells. Individual pancreata were perfused with PBS. After removal of all visible pancreatic lymph nodes, the pancreata were digested in HBSS containing 5 mg/ml collagenase IV and 2.5 mg/ml DNase (Sigma Chemical Co.), by shaking (200 rpm) at 37°C for 12 min. Single cell suspensions were

collected after diluting the enzymes with ice-cold HBSS containing 5% FCS and removal of the aggregates by settling 5 min on ice. Aggregates were further digested with collagenase IV (2.5 mg/ml) and DNase (1.25 mg/ml) for 5 min. Single cell suspensions from three to four mice were pooled, washed three times, and $CD4^+$ and $CD8^+$ cells were sorted by positive selection on Mini-MACS® (Miltenyi Biotec Inc., Sunnyvale, CA). The mean number of $CD4^+$ and $CD8^+$ cells recovered per pancreas in three separate experiments was, respectively, 51 and 20 × 10^4 for vehicle-injected mice, 82 and 66 × 10^4 for IL-12–treated littermates.

Induction of Lymphokine Production. $CD4^+$ and $CD8^+$ cells were cultured (2.5 × 10^5 cells/well) in 96-well round bottom plates coated with 5 μg/ml anti-TCR mAb (American Type Culture Collection [ATCC] HB 218; Rockville, MD) in serum-free HL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin (Sigma Chemical Co.). Supernatants were collected after 48 h of culture. Sera were collected ~15 h after the last IL-12 injection and assayed for ELISA for circulating IFN-γ.

Immunohistology. For hematoxylin and eosin staining, pancreata were fixed in 10% formalin and embedded in paraffin. For immunoperoxidase staining, pancreata were snap-frozen in Tissue-Tek (Miles Laboratories Inc., Elkhart, IN), and consecutive 5 μm sections stained for 45 min with rabbit anti–glucagon and mouse anti–insulin antibodies (Ortho Diagnostic Systems, Raritan, NJ). After washing, sections were incubated for 30 min with secondary antibodies conjugated to horseradish peroxidase, which was visualized using 3 amino-9 ethyl-carbazole (AEC, Sigma Chemical Co.) as chromogen and hematoxylin as counterstain. In addition, pancreas cryostat sections were stained by biotinylated mAb directed against CD4, CD8, B220 (PharMingen, San Diego, CA), p150/90 leucocyte β2 integrin (N418, ATCC), and $I\text{-}A^{g7}$ (10.3.62, ATCC), followed by streptavidin–peroxidase conjugate. AEC was used as a chromogen and hematoxylin as a counterstain.

Cytokine Assays. IFN-γ and IL-4 concentrations were determined by ELISA using, respectively, mAb AN-18.17.24 (22) or BVD4-1D11 (PharMingen) for capture, and peroxidase-conjugated XMG1.2 (23) or biotinylated BVD6-24G2 (PharMingen) followed by avidin-peroxidase (Sigma Chemical Co.) for detection. The substrate was 3,3′-5,5′-tetramethylbenzidine (Fluka Chemie AG, Buchs, Switzerland). Dilutions of culture supernatant or serum were assayed in the linear portion of the dose–response curve. Standard curves were generated in each assay using purified recombinant mouse IFN-γ or IL-4.

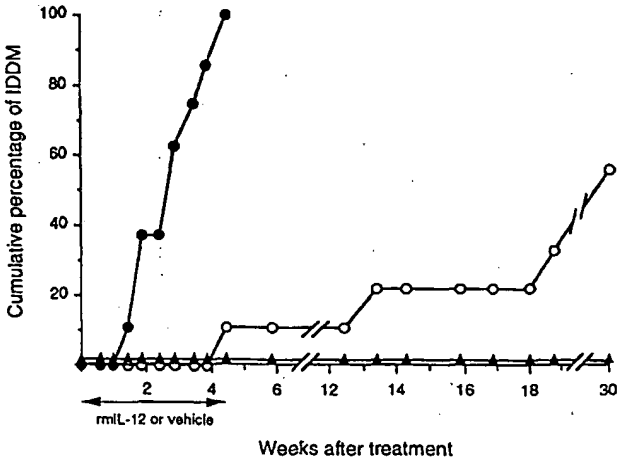

Figure 1. Acceleration of IDDM onset by IL-12 administration to NOD mice. 10 female NOD/Lt prediabetic mice (8–10 wk old, randomized from four different litters) were injected daily with 0.3 μg i.p. recombinant mouse IL-12 for the first 7 d, then with 0.15 μg for the following 24 d (●), or with vehicle (PBS containing 1% NOD serum) only (○). As control, 10 BALB/c female mice (10 wk old) received the same IL-12 treatment (▲). Four other independent experiments, performed with 10–14-wk-old NOD females injected with 0.15 μg IL-12 daily, gave similar results, IDDM onset starting after 5–10 IL-12 injections.

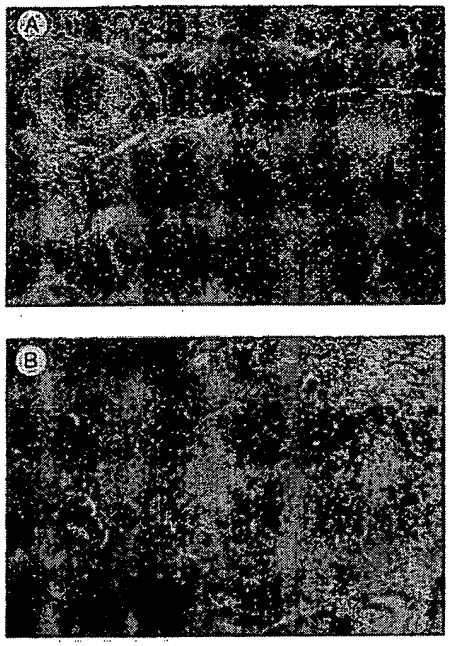

Figure 2. Insulitis is observed in NOD but not in BALB/c mice after administration of IL-12. Islets from a 10-wk-old BALB/c mouse after 32 injections of IL-12 are intact (*A*). In contrast, islets from a 10-wk-old NOD mouse after 20 injections of IL-12 show a severe mononuclear cell infiltration (*B*). ×250.

Results and Discussion

Administration of recombinant mouse IL-12 to 8–10-wk-old prediabetic NOD female mice induced rapid onset of IDDM (Fig. 1). Overt diabetes began after about 10 IL-12 injections, and after 30 injections it was present in 100% of the mice. The same treatment did not induce IDDM in BALB/c mice. In the NOD female littermates injected with vehicle only (PBS-1% normal NOD serum), IDDM began only at 15 wk of age, and by week 40, hyperglycemia was present in 55% of the mice.

Histological analysis of pancreatic sections revealed insulitis in NOD mice injected with IL-12, but not in BALB/c mice, even after 32 injections of IL-12 (Fig. 2). The normal appearance of pancreatic islets and the absence of IDDM in BALB/c mice indicates a lack of direct toxic effects of IL-12 administration on islet β cells in vivo. Quantification of islet infiltrates showed a higher score in NOD mice injected with IL-12 as compared with those injected with vehicle only (not

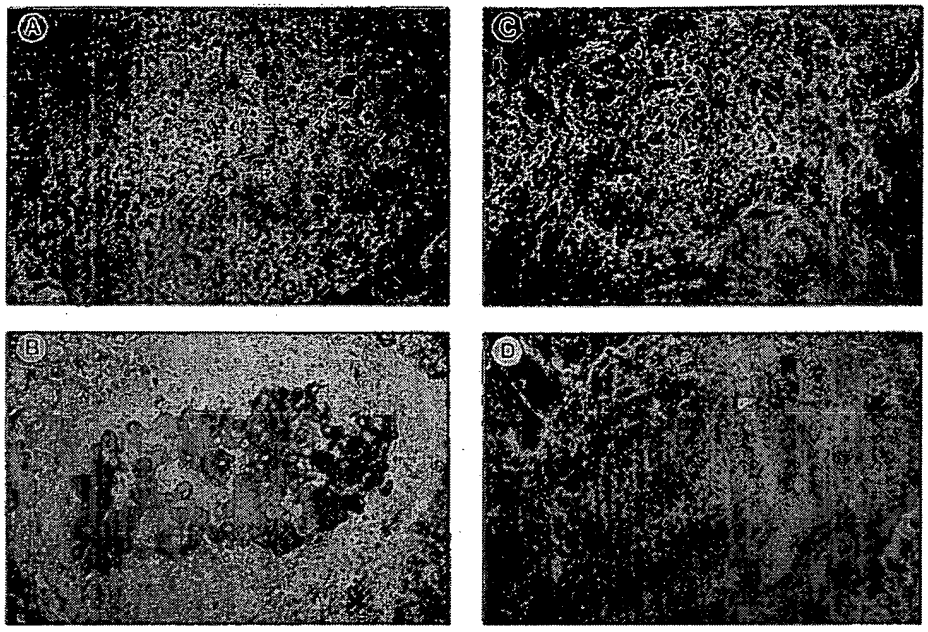

Figure 3. Selective loss of islet β cells in NOD mice injected with IL-12. Pancreas from a 10-wk-old NOD female mouse injected 14 times with vehicle only displays insulitis, but glucagon- (*A*) and insulin- (*B*) containing islet cells are well represented. In contrast, the NOD littermate injected 14 times with IL-12 (0.15 μg/day) had disorganized but relatively normal numbers of glucagon-containing cells (*C*), whereas insulin-containing cells (*D*) are absent. The latter finding correlates with hyperglycemia (>500 mg/dl) at the time of pancreas removal. ×400.

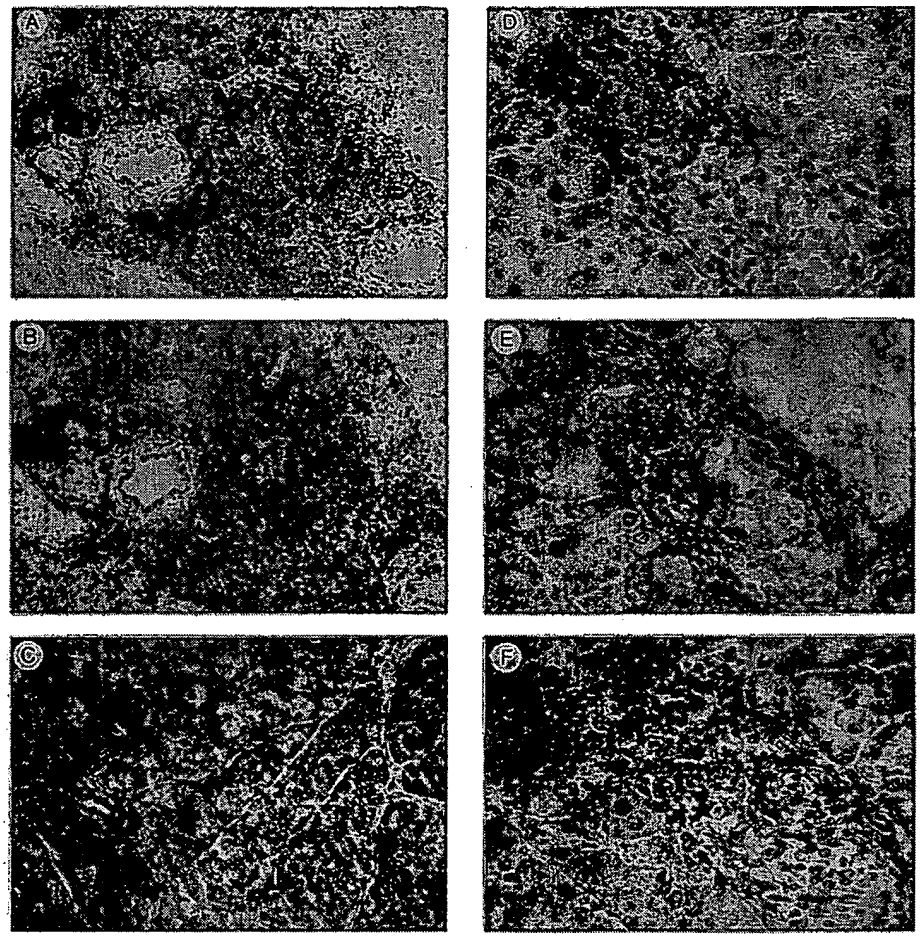

Figure 4. Immunostaining of pancreas-infiltrating cells in NOD mice injected with IL-12. Massive infiltration containing high numbers of CD4$^+$ (*A*), and CD8$^+$ (*B*) T cells is observed in the pancreas from a 10 wk-old NOD female injected 14 times with IL-12 (0.15 μg/day). An infiltrated islet from this mouse shows abundant B220$^+$ (*D*), class II$^+$ (*E*) and N418$^+$ (*F*) cells. N418$^+$ cells are also present in the exocrine pancreas (*C*). *A–C*, ×200; *D–F*, ×400.

Table 1. *Production of IFN-γ and IL-4 by Pancreas-infiltrating CD4+ or CD8+ T Cells from NOD Mice*

	IFN-γ						IL-4			
			Pancreas-infiltrating cells				Pancreas-infiltrating cells			
	Serum		CD4+		CD8+		CD4+		CD8+	
	Day 7	Day 14	–	α-TCR	–	α-TCR	–	α-TCR	–	α-TCR
Vehicle	<5	<5	<15	9,241	<15	9,200	148	2,268	50	107
IL-12	303	125	<15	37,226	<15	17,721	59	471	<15	<15

Pancreas-infiltrating CD4+ or CD8+ cells from 9-wk old NOD mice injected with IL-12 (0.15 μg/mouse/d for 13–14 d) or with vehicle only were stimulated with insolubilized anti-TCR mAb (5 μg/ml). After 48 h of culture, IFN-γ and IL-4 production (pg/ml) were quantified by ELISA. Sera were assayed by ELISA for circulating IFN-γ (pg/ml). Results are means of two to four experiments.

shown). IL-12–treated mice had profoundly reduced numbers of insulin-secreting β cells, whereas glucagon-producing cells were not affected (Fig. 3). These results demonstrate that the diabetes induced by IL-12 administration is of an autoimmune nature, selectively destroying insulin-producing β cells. Surface markers of pancreas-infiltrating cells were analyzed by immunohistochemistry and representative sections from IL-12–injected NOD mice are shown in Fig. 4. Islet infiltrates are dominated by CD4+ and CD8+ T cells and tend to extend into the exocrine pancreas (*A* and *B*). IL-12 administration induces an increase in the number of CD4+ cells, and a marked increase of CD8+ cells (see Materials and Methods). The infiltrates contain class II+ cells (*E*) which include B cells (*D*) and N418+ cells (*F*). The latter cells are abundant also in the exocrine pancreas (*C*). The N418 antibody recognizes CD11c, an integrin expressed on dendritic cells and at a low level on macrophages in normal lymphoid tissues (24). Thus, all the cell types necessary for an immune response are present in the islets of IL-12–treated mice.

To determine whether IL-12 administration induces Th1 cells we tested IFN-γ and IL-4 production by islet-infiltrating T cells after stimulation by insolubilized anti-TCR mAb. Islet-infiltrating CD4+ cells from NOD mice injected with IL-12 produced high amounts of IFN-γ, about fourfold higher than T cells from NOD mice injected with vehicle only (Table 1). Conversely, IL-4 production was decreased in pancreas-infiltrating CD4+ cells from IL-12 treated as compared with control NOD mice. These results indicate that CD4+ cells infiltrating the lesion site in IL-12–injected NOD mice mostly exhibit a Th1 lymphokine profile. Pancreas-infiltrating CD8+ cells, which participate in IDDM induction (25), also produce high levels of IFN-γ and little IL-4, indicating a Th1-like phenotype (26). IFN-γ could also be detected in the serum of NOD mice injected with IL-12 (Table 1). In agreement with previous results, it peaked 1 wk after beginning of IL-12 administration (21).

These results show that administration of IL-12 accelerates the onset of IDDM and induces disease in all NOD female mice tested, whereas only ~60% of control littermates eventually develop IDDM. Acceleration of IDDM is accompanied by expansion of Th1 cells and by selective destruction of islet β cells, suggesting a causal link between IL-12, Th1 cell induction, and development of IDDM. The spontaneous development of IDDM may also involve IL-12–dependent generation of Th1 cells. This possibility will be tested by administration of IL-12 antagonists (27) to NOD mice, a potential therapeutic approach for the treatment of IDDM and of other Th1 cell–mediated autoimmune diseases. The present results also suggest that IL-12, a possible therapeutic agent against tumors (28) and infectious diseases (29), should be administered cautiously to patients with Th1-mediated autoimmune diseases, or with a known predisposition to them.

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References

1. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffmann. 1986. Two types of murine helper T cell clone. I Definition according to profile of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348–2357.
2. Del Prete, G., M. De Carli, C. Mastromauro, R. Biagiotti, D. Macchia, P. Falagiani, M. Ricci, and S. Romagnani. 1991. Purified protein derivative of mycobacterium tuberculosis and excretory-secretory antigen(s) of toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J. Clin. Invest.* 88:346–350.
3. Trinchieri, G. 1993. Interleukin-12 and its role in the generation of Th1 cells. *Immunol. Today.* 14:335–338.
4. Paul, W.E., and R.A. Seder. 1994. Lymphocytes responses and cytokines. *Cell.* 76:241–251.
5. Wolf, S.F., P.A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, D. Ferenz, R.M. Hewick, et al. 1991. Cloning of cDNA for natural killer stimulatory factor, a heterodimeric cytokine with multiple biological effects on T and natural killer cells. *J. Immunol.* 146:3074–3081.
6. Gubler, U., A.O. Chua, D.S. Schoenhaut, C.M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A.G. Wolitzky, P.M. Quinn, P.C. Familletti, and M.K. Gately. 1991. Coexpression of two distinct genes is required to generate secreted, bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA.* 88:4143–4147.
7. D'Andrea, A., M. Rengaraju, N.M. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg, et al. 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176:1387–1398.
8. Kobayashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170:827–845.
9. Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Pospíšil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark, and G. Trinchieri. 1991. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 173:869–879.
10. Debray-Sachs, M., C. Carnaud, C. Boitard, H. Cohen, I. Gresser, P. Bedossa, and J.-F. Bach. 1991. Prevention of diabetes in NOD mice treated with antibody to murine IFN-γ. *J. Autoimmun.* 4:237–248.
11. Campbell, I.L., T.W. Kay, L. Oxbrow, and L.C. Harrison. 1991. Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J. Clin. Invest.* 87:739–742.
12. Sarvetnick, N., D. Liggitt, S.L. Pitts, S.E. Hansen, and T.A. Stewart. 1988. Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. *Cell.* 52:773–782.
13. Kaufman, D.L., M. Clare-Salzler, J. Tian, T. Forsthuber, G.S.P. Ting, P. Robinson, M.A. Atkinson, E.E. Sercarz, A.J. Tobin, and P.V. Lehmann. 1993. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature (Lond.).* 366:69–72.
14. Tisch, R., X.-D. Yang, S.M. Singer, R.S. Liblau, L. Fugger, and H.O. McDevitt. 1993. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature (Lond.).* 366:72–75.
15. Rapoport, M.J., A. Jaramillo, D. Zipris, A.H. Lazarus, D.V. Serreze, E.H. Leiter, P. Cyopick, J.S. Danska, and T.L. Delovitch. 1993. Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. *J. Exp. Med.* 178:87–99.
16. Pennline, K.J., E. Roquegaffney, and M. Monahan. 1994. Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clin. Immunol. Immunopathol.* 71: 169–175.
17. Scott, B., R. Liblau, S. Degermann, L.A. Marconi, L. Ogata, A.J. Caton, H.O. McDevitt, and D. Lo. 1994. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. *Immunity.* 1:1–20.
18. Manetti, R., P. Parronchi, M.G. Giudizi, M.-P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4–producing Th cells. *J. Exp. Med.* 177:1199–1204.
19. Hsieh, C.-S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of Th1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science (Wash. DC).* 260:547–549.
20. Afonso, L.C.C., T.M. Scharton, L.Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott. 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major. Science (Wash. DC).* 263:235.
21. Gately, M.K., R.R. Warrier, S. Honasoge, D.M. Carvajal, D.A. Faherty, S.E. Connaughton, T.D. Anderson, U. Sarmiento, B.R. Hubbard, and M. Murphy. 1994. Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-γ in vivo. *Int. Immunol.* 6:157–167.
22. Prat, M., G. Gribaudo, P.M. Comoglio, G. Cavallo, and S. Landolfo. 1984. Monoclonal antibodies against murine γ interferon. *Proc. Natl. Acad. Sci. USA.* 81:4515–4519.
23. Cherwinski, H., J.H. Shumacher, K.D. Brown, and T.R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* 166: 1229–1244.
24. Crowley, M.T., K. Inaba, M.D. Witmer-Pack, S. Gezelter, and R.M. Steinman. 1990. Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. *J. Immunol. Methods.* 133:55–66.
25. Bendelac, A., C. Carnaud, C. Boitard, and J.-F. Bach. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4$^+$ and Lyt-2$^+$ T cells. *J. Exp. Med.* 166:823–832.
26. Erard, F., M.-T. Wild, J.A. Garcia-Sanz, and G. Le Gros. 1993. Switch of CD8 T cells to noncytolytic $CD8^-CD4^-$ cells that make T_H2 cytokines and help B cells. *Science (Wash. DC).* 260:1802–1805.
27. Mattner, F., S. Fischer, S. Guckes, S. Jin, H. Kaulen, E. Schmitt, E. Rude, and T. Germann. 1993. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *Eur. J. Immunol.* 23:2202–2208.
28. Brunda, M.J., L. Luistro, R.R. Warrier, R.B. Wright, B.R. Hubbard, M. Murphy, S.F. Wolf, and M.K. Gately. 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J. Exp. Med.* 178:1223–1230.
29. Heinzel, F.P., D.S. Schoenhaut, R.M. Rerko, L.E. Rosser, and M.K. Gately. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major. J. Exp. Med.* 177:1505–1509.

ed to speak about the social and psychological circumstances of his patient. It all seemed a mere distraction, so tangential to the pathogenesis and clinical management.

As more and more friends and family members of my generation became ill, the convenient illusion that there is a wide gulf between physician and patient was eroding. But to abandon this illusion would be detrimental, because it permits us to stand at the bedside without flinching at some of the most gruesome and threatening maladies that afflict men and women. As doctors age, I suspect that our shift of focus from the purely clinical to a perspective incorporating the emotional and spiritual reflects the realization that our powers are limited, that in the midst of human biology, we are also seeking knowledge about resilience and courage, attributes that we hope will be ours when disease strikes us.

A few months after my conversation with the hematology fellow, I was sitting at dinner with Pam, as we reviewed with each other the events of our day. "I was referred a great case," she said. I was taken aback by her use of the phrase. She registered my reaction, but she continued. "A woman in her late 50s, with a blood pressure of 240 over 120, who had been dizzy and flushing. One doctor concluded that she was hypertensive and beginning menopause and prescribed standard medications for her blood pressure, including an ACE inhibitor. Then a consulting nephrologist, concerned about her kidneys, ordered an abdominal scan. And there it was: an adrenal mass. It turned out to be a pheochromocytoma."

It had, I told myself, key elements of "a great case": the initial misdiagnosis, the confluence of disparate symptoms and signs of an unusual disease, the instance when standard therapies can be paradoxically harmful, the complex coordination of medical and surgical management. But still I resisted the appellation.

"What makes it a great case?" I asked.

Pam replied that it had provoked an animated discussion among the students and house staff, that important teaching points had been made. But then she reflected more deeply. "A great case because you not only make the diagnosis — you do something fundamental about it. You can really help."

This conception came closer to the equipoise that had been eluding me. It was foolish to deny the profound intellectual excitement that came from medicine. It was also a hollow form of medicine that was practiced without factoring in the ultimate outcome. But that balance required exceptional circumstances. It necessitated knowing the outcome and could therefore be experienced only with hindsight, whereas thoughts and feelings are sparked from the start and demand a language right at the outset if they are to be shared.

I still find myself unable, except in retrospect, to retrieve the language of my youth and speak about "a great case." It is as if medicine at this stage of my life has split into two streams — a current of marvelous biology and an undertow that pulls at the soul. From the bank where I stand, it is hard to imagine that these two streams can ever again flow as one.

From the Beth Israel Deaconess Medical Center and Harvard Medical School, Boston.

Cytokine-Based Therapies for Crohn's Disease — New Paradigms

Fabio Cominelli, M.D., Ph.D.

Related article, page 2069

Crohn's disease is one of two idiopathic inflammatory bowel diseases that affect approximately 1 million people in North America. Despite important advances in diagnosis and treatment in recent years, the underlying cause of the disease remains unclear. As a result, the available therapies are not curative and may pose a substantial risk of side effects. A current theory regarding the pathogenesis of Crohn's disease suggests that there is an overly aggressive immune response against com-

mensal bacteria in a genetically predisposed person. In this context, the activation of lymphocytes and the overexpression of inflammatory cytokines represent a common effector mechanism leading to chronic intestinal inflammation. This theory has led to the development of several novel therapeutic agents that specifically block the production of key cytokines in patients with Crohn's disease, including the first successful therapeutic biologic agent, infliximab. However, this anti–tumor necrosis factor α (TNF-α) agent is far from being universally applicable and has raised as many questions as it has provided solutions.

The classic paradigm for cytokine involvement in the pathogenesis of Crohn's disease focuses on type 1 helper T-cell (Th1) cytokines, such as TNF-α, interleukin-12, and interferon-γ, which are thought to have a primary role in initiating the disease process. Conversely, type 2 helper T-cell (Th2) cytokines, such as interleukin-4 and interleukin-13, are considered to have a more prominent role in ulcerative colitis, the other idiopathic inflammatory bowel disease. However, mounting evidence suggests that this classic Th1–Th2 paradigm may be overly simplistic, and the hypothesis that these two pathways are always mutually exclusive has recently been challenged. Immunologic models are therefore now being proposed that involve both clusters of cytokines. Furthermore, individual cytokines may have diverse, and even opposing, functions in different clinical and immunologic scenarios. This is particularly true in Crohn's disease, in which the inflammatory process appears to develop in two distinct phases — an initial, inductive phase and an effector phase characterized by chronic inflammation. Both Th1 and Th2 pathways may be involved in each phase, either concomitantly or sequentially. Taking into account all these observations, some have suggested that a distinction between innate and adaptive cytokines may be more accurate than the distinction between Th1 and Th2 cytokines (see Figure).[1]

The traditional viewpoint has been that Crohn's disease results from a dysregulated response by the acquired immune system. However, recent evidence indicates that the innate immune system may be equally important, especially in the inductive phase of disease. First, it is now recognized that defects in epithelial barrier function, such as those that are present in a variety of animal models, lead to intestinal inflammation. Second, cells of the innate immune system are important producers of cytokines such as interleukin-1, TNF-α, and interleukin-6, which have important inflammatory effects within the intestinal mucosa. Finally, the first gene that has been described as conferring susceptibility to Crohn's disease encodes an intracellular protein (i.e., NOD2/CARD15) used by cells of the innate immune system to sense the presence of bacterial products through the activation of nuclear factor-κB–dependent cytokines. Despite the fact that the precise function of NOD2/CARD15 during intestinal inflammation remains unclear, these observations have led to the intriguing hypothesis that Crohn's disease may be initiated by a defective innate immune response, with decreased production of nuclear factor-κB–dependent cytokines in response to commensal bacteria. In this regard, it could be argued that blocking certain cytokines in the initial phases of Crohn's disease may not be beneficial and could actually aggravate the existing disease process.

Several inflammatory cytokines have been targeted for therapeutic intervention in Crohn's disease; these include interleukins 1, 2, 6, 12, and 18, interferon-γ, and TNF-α (see Figure). The most successful example of such intervention to date has been the use of anti–TNF-α therapy in patients with refractory and fistulizing Crohn's disease. In this issue of the *Journal*, Mannon et al. (pages 2069–2079) report on the safety of an anti–interleukin-12 monoclonal antibody administered subcutaneously for seven weeks. Despite the small number of patients included in this study, the authors describe a significant clinical response in the group that received the higher dose (3 mg per kilogram of body weight).

Like TNF-α, interleukin-12 is an important Th1-polarizing cytokine that is strongly implicated in the pathogenesis of Crohn's disease. In addition to neutralizing the inflammatory effects of TNF-α and interleukin-12, anti–TNF-α and anti–interleukin-12 therapies appear to share a common mechanism of inducing apoptosis in activated lymphocytes that have infiltrated the intestinal mucosa.[2-4] Protection of intestinal epithelial cells from apoptosis has also been suggested as a potential anti-inflammatory mechanism of anti–TNF-α therapy in Crohn's disease.[3]

Given these early successes, more cytokines are rapidly being identified as potential therapeutic tar-

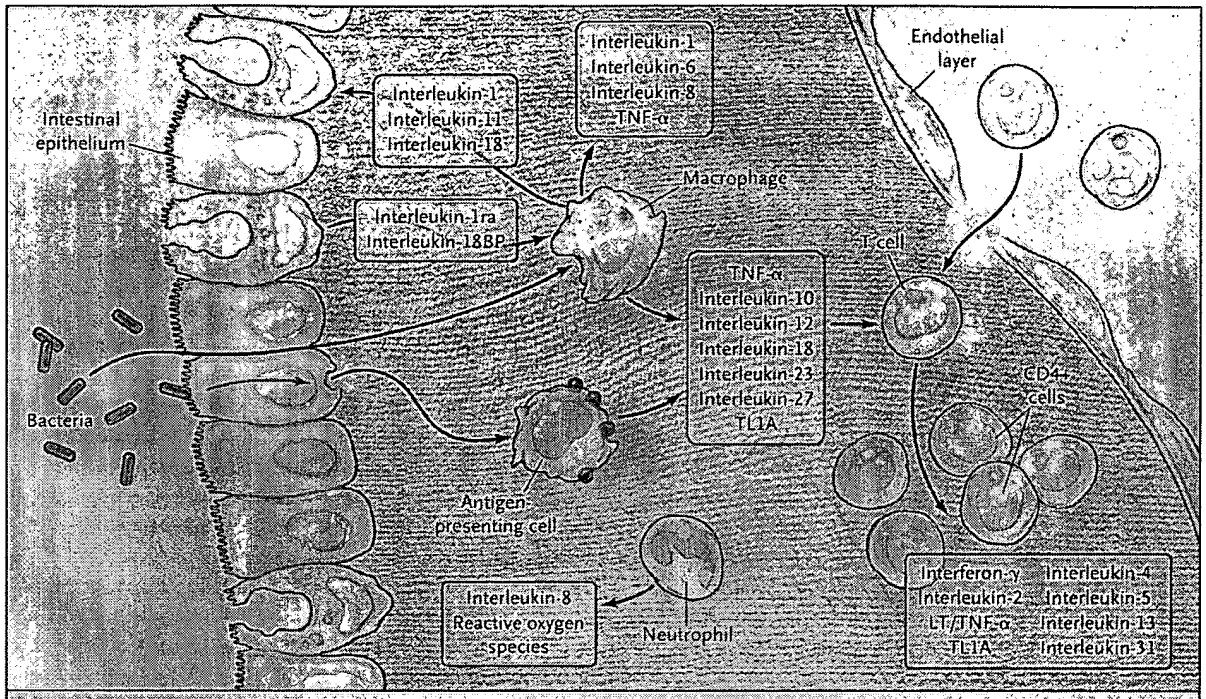

Figure. Working Hypothesis Regarding the Role of Cytokines in the Pathogenesis of Crohn's Disease.
When the mucosal immune system in patients predisposed to the development of Crohn's disease is first exposed to an initiating antigenic stimulus, a dysregulated and overly aggressive cytokine-mediated T-cell response is mounted. Cytokines involved in innate immune responses, such as tumor necrosis factor α (TNF-α), interleukin-1, interleukin-6, and possibly interleukins 12 and 18, may play a key role in this phase. Once CD4+ T cells are activated, effector cytokines involved in the adaptive immune response, including TNF-α and interferon-γ, as well as interleukins 4 and 13, mediate the effector phase of the intestinal inflammatory response. Novel cytokines such as TL1A and interleukins 23, 27, and 31 may also contribute to the effector phase. BP denotes binding protein, ra receptor antagonist, ROS reactive oxygen species, and LT lymphotoxin.

gets; among them are interleukins 18, 23, 27, and 31, as well as TL1A. In addition, the therapeutic potential of cytokines that belong to the traditional Th2-polarizing class is being evaluated; this group includes interleukins 4 and 13, for which there is increasing evidence of a potential inflammatory role, particularly in small intestinal disease.

Cytokine blockade with monoclonal antibodies, fusion proteins, and receptor antagonists remains an attractive method of immunomodulation in Crohn's disease. However, important questions remain regarding the safety of long-term treatment, especially since classic Th1 cytokines are important in combating infections. The reactivation of latent tuberculosis in patients who have received anti–TNF-α therapy is an example of the potential for harm due to the long-term suppression of host responses against certain infectious agents.[5] The long-term risk of malignancy with anti-cytokine therapeutics in general and the possibility that anti–interleukin-12 therapy, in particular, may reactivate asthma also need to be considered. In addition, on the basis of our improved understanding of the complex pathogenesis of Crohn's disease — in particular, the existence of multiple phases of disease — the realization is developing that blocking certain cytokines may be detrimental, rather than beneficial, in some situations. The inability of anti–TNF-α treatment (infliximab) to induce remission in a substantial percentage of patients with Crohn's disease could indicate that non-Th1 effector pathways or a different disease phase may predominate in these patients. An effective treatment strategy for such patients might therefore involve the block-

ade of multiple cytokines in order to intervene in several pathways. Despite these limitations, the development of anticytokine therapies as part of the physician's armamentarium is an important staging post on the road to a cure for Crohn's disease.

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1. Reuter BK, Pizarro TT. The role of the IL-18 system and other members of the IL-1R/TLR superfamily in innate mucosal immunity and the pathogenesis of inflammatory bowel disease: friend or foe? Eur J Immunol 2004;34:2347-55.

2. Van den Brande JM, Braat H, van den Brink GR, et al. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology 2003;124:1774-85.

3. Marini M, Bamias G, Rivera-Nieves J, et al. TNF-alpha neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis. Proc Natl Acad Sci U S A 2003;100:8366-71.

4. Fuss IJ, Marth T, Neurath MF, Pearlstein GR, Jain A, Strober W. Anti-interleukin 12 treatment regulates apoptosis of Th1 T cells in experimental colitis in mice. Gastroenterology 1999; 117:1078-88.

5. Tuberculosis associated with blocking agents against tumor necrosis factor-alpha — California, 2002–2003. MMWR Morb Mortal Wkly Rep 2004;53:683-6.

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Immunology

Increased interleukin 12 production in progressive multiple sclerosis: Induction by activated CD4$^+$ T cells via CD40 ligand

(autoimmunity/gp39/interferon-γ)

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ABSTRACT **Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system postulated to be a cell-mediated autoimmune disease in which interferon γ (IFN-γ) plays an important role. There is increased IFN-γ secretion in MS, and IFN-γ administration induces exacerbations of disease. We found that interleukin 12 (IL-12) was responsible for raised IFN-γ secretion in MS as anti-IL-12 antibodies reversed raised anti-CD3-induced IFN-γ in MS patients to normal levels. Furthermore, we found a marked increase in T cell receptor-mediated IL-12 secretion in progressive MS patients vs. controls (24.8 ± 7.7 pg/ml vs. 1.5 ± 1.0 pg/ml, $P = 0.003$) and vs. relapsing–remitting patients (3.7 ± 1.4 pg/ml, $P < 0.05$). Investigation of the cellular basis for raised IL-12 demonstrated that T cells from MS patients induced IL-12 secretion from non-T cells, and that T cells from MS patients could even drive non-T cells from normal subjects to produce increased IL-12. Anti-CD40 ligand antibody completely blocked IL-12 secretion induced by activated T cells, and we found increased CD40 ligand expression by activated CD4$^+$ T cells in MS patients vs. controls. The CD40 ligand-dependent Th1-type immune activation was observed in the progressive but not in the relapsing–remitting form of MS, suggesting a link to disease pathogenesis and progression and providing a basis for immune intervention in the disease.**

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system postulated to be a T cell-mediated autoimmune disease (1). Interferon γ (IFN-γ), a cytokine that is the hallmark of Th1-type immune responses, plays an important role in disease pathogenesis as increased production of IFN-γ precedes clinical attacks (2, 3), and injection of MS patients with recombinant IFN-γ-induced exacerbations of the disease (4). Furthermore, within the nervous system, the inflammatory process is characterized by increased IFN-γ expression (5). Little is known about the immune basis for raised IFN-γ in MS or how it relates to different stages of the disease. Interleukin 12 (IL-12), a cytokine produced by non-T cells, is the most potent inducer of IFN-γ and Th1-type immune responses (6). We recently observed increased expression of IL-12p40 mRNA associated with inflammation in the central nervous system of MS patients but not in patients suffering from stroke (7). Given the importance of IL-12 in IFN-γ induction, we investigated IFN-γ secretion in MS and its potential link to IL-12.

MATERIALS AND METHODS

Subjects. MS patients were studied from the outpatient MS clinic of the Brigham and Women's Hospital. Relapsing–remitting patients ($n = 18$; average age = 44 ± 1.3 years) had an average expanded disability status (EDSS) of 3.9 ± 0.5, and chronic progressive MS patients ($n = 33$; average age = 46 ± 1.1 years) had an EDSS of 5.7 ± 0.3. A disability of 6 or greater involves use of a cane or other support. Patients had not received immunosuppressive therapy in the past or steroid treatment in the 6 months before blood drawing. The control group consisted of age and sex matched healthy subjects ($n = 29$; average age = 43 ± 1.8 years). The number of patients used for each individual experiment is given in the corresponding table or figure legends.

Cell Separation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll/Hypaque density gradient centrifugation (Pharmacia LKB). Cells were resuspended (10^6 cells/ml) in complete culture media consisting of RPMI 1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM Hepes buffer, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from BioWhittaker). Separation of T cells from PBMC was performed by using negative depletion of non-T cells with human T cell enrichment column (R & D Systems) according to manufacturer's instructions. Separation of non-T cells (antigen-presenting cells; APCs) from PBMC was done by using negative depletion of T cells with Dynabeads M-450 Pan T (CD2) (Dynal; Great Neck, NY) according to manufacturer's instructions. Separation of T cells into CD4 depleted (CD4$^-$) and CD8 depleted (CD8$^-$) T cells was performed using Dynabeads M-450 CD4 and Dynabeads M-450 CD8, respectively (Dynal).

Cell Culture. *PBMC activation with soluble anti-CD3.* In preliminary experiments during which culture conditions were established, we found that following 2 days of *in vitro* culture, prominent IFN-γ secretion was observed with anti-CD3 mAb stimulation but not with IL-2 or IL-12. Thus to study the T cell receptor complex (TcR)-mediated pathway of IFN-γ secretion, we chose culture conditions in which 1 ml of PBMC (1×10^6 cells) was placed in polypropylene culture tubes (Fisher Scientific), cultured for 2 days in medium, washed, and then activated with 1 μg/ml of anti-CD3 mAb (American Type Culture Collection; clone OKT3, mouse IgG2a) and culture supernatants were collected 24 or 48 h later. Activated cells were also used for flow cytometry analysis of IL-12 receptor (IL-12R) positive cells.

T cell activation with immobilized anti-CD3. T cells (1×10^6 cells/well) and/or APCs (5×10^6 cells/well) were placed in the total volume of 1 ml in the wells of a 24-well flat-bottom plate

Abbreviations: MS, multiple sclerosis; IFN-γ, interferon γ; PBMC, peripheral blood mononuclear cells; EAE, experimental autoimmune encephalomyelitis; APC, antigen presenting cell; Ab, antibody; FITC, fluorescein isothiocyanate; IL, interleukin; IL-12R, IL-12 receptor; rIL-12, recombinant IL-12; TNF-α, tumor necrosis factor α; TcR, T cell receptor.

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with immobilized anti-CD3 or with immobilized control mouse IgG2a. Culture supernatants were collected after 24-h incubation. Activated T cells were also studied for CD40 ligand expression by flow cytometry after 20-h incubation and at various other times for kinetic studies.

Cytokine ELISA. IFN-γ and IL-4 determinations in culture supernatants was performed by ELISA using a cytokine ELISA protocol from PharMingen. For IFN-γ and IL-4, 1 μg/ml of capture mouse anti-human IFN-γ mAb or mouse anti-human IL-4 mAb, and 1 μg/ml of biotinylated mouse anti-human IFN-γ mAb or biotinylated rat anti-human IL-4 mAb were used (all from PharMingen). IL-12p70 and IL-2 were determined by appropriate ELISA kits (R & D Systems and BioSource International, Camarillo, CA, respectively). For standards, recombinant human IFN-γ (GIBCO/BRL), recombinant human IL-2 (Boehringer Mannheim), recombinant human IL-4 (PharMingen), and recombinant human IL-12 (R & D Systems) were used. Sensitivity of IFN-γ, IL-2, IL-4, and IL-12 ELISA were 32, 32, 8, and 2 pg/ml, respectively.

Flow Cytometry. IL-12R-bearing cells were detected as described by Desai *et al.* (8). Briefly, 1×10^6 cells in 0.1 ml staining buffer (PBS/2% fetal calf serum/0.1% sodium azide) were sequentially incubated with 40 nM unlabeled IL-12 for 40 min, followed by biotinylated rat anti-human IL-12 (Clone 4D6, IgG1, provided by M. K. Gately (Hoffmann–La Roche) or control biotinylated rat IgG1 (clone MP4-25D2; PharMingen) for 20 min, and finally with Streptavidin–phycoerythrin for 20 min. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated mAb specific for CD3, CD16 (both from AMAC, Westbrook, ME), CD4, CD8, or control FITC-conjugated mouse Ig (all from Coulter Immunology) according to the manufacturer's recommendations). T cells activated with immobilized anti-CD3 were stained with FITC-conjugated mAb specific for CD4 (Coulter Immunology) and phycoerythrin-conjugated anti-CD40 ligand mAb (PharMingen) or control FITC- and phycoerythrin-conjugated mouse Ig (Coulter Immunology) according to the manufacturer's recommendations. All incubations were carried out at 4°C in staining buffer, and cells were washed twice between incubations. Flow cytometric analysis of $5–10 \times 10^3$ cells from each sample was performed on an FACSort flow cytometer (Beckton Dickinson) according to standard procedures.

Statistical Analysis. Results are presented as mean ± SEM for each group. Statistical significance was calculated using Student's *t* test.

RESULTS AND DISCUSSION

To investigate the basis for raised IFN-γ production in MS, we stimulated T cells through the TcR with anti-CD3 mAb. We first studied a group of chronic progressive MS patients and healthy control subjects and found a clear increase in TcR-mediated IFN-γ production in MS. Specifically, anti-CD3-induced IFN-γ production was almost 3-fold higher in progressive MS ($n = 25$) than in control subjects ($n = 21$), 2132 ± 207 pg/ml in MS vs.756 ± 121 pg/ml in controls, $P < 0.001$.

To investigate the possible role of IL-12 in the raised IFN-γ secretion in progressive MS, we added anticytokine neutralizing antibody (Ab) to cultures stimulated with anti-CD3. In addition to progressive MS, we also studied patients with relapsing–remitting disease. Clinically, a classic feature of MS is a course that initially involves relapses followed by remission of disease (9). The relapsing–remitting phase is often followed by a progressive phase that involves progressive neurologic deterioration and is a major source of disability in MS. Some patients have progressive disease from the onset. Why the character of the illness changes from relapsing-remitting to progressive is unknown. As shown in Fig. 1 *Top*, an increase in anti-CD3-induced IFN-γ production was seen in patients with

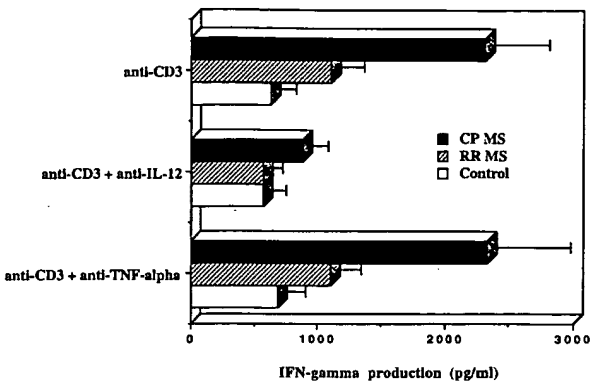

FIG. 1. Increased TcR-mediated IFN-γ production in MS is linked to defective regulation by endogenous IL-12. PBMC (1×10^6/ml) from controls, relapsing–remitting MS (RR MS), or chronic progressive MS (CP MS) were stimulated with anti-CD3 mAb as described. Neutralizing goat anti-human IL-12 (10 μg/ml, R & D Systems) or isotype control goat anti-human tumor necrosis factor α (TNF-α; 10 μg/ml, R & D Systems) were added to the cultures. After 2 days the levels of IFN-γ production in culture supernatants were measured by ELISA; data are expressed in pg/ml. Data are mean ± SEM from tested control subjects ($n = 10$), relapsing–remitting MS ($n = 9$), and chronic progressive MS ($n = 11$) patients. IFN-γ production in CP MS was significantly higher then in control subjects ($P < 0.005$) or relapsing–remitting MS ($P < 0.05$). Neutralizing anti-IL-12 significantly reduced IFN-γ production in chronic progressive MS ($P = 0.009$) to the level of control subjects. T cell proliferative responses or IL-4 production in MS patients or in control subjects were not affected by anti-IL-12. Isotype control mouse IgG2a did not induce any detectable level of IFN-γ or IL-4.

the chronic progressive form of MS but only minimally in relapsing–remitting subjects. In addition (Fig. 1 *Middle*), anti IL-12 Ab reversed the elevated anti-CD3-induced IFN-γ secretion in chronic progressive MS to the level of control subjects ($P = 0.009$). Anti-TNF-α did not affect anti-CD3-induced IFN-γ production in either MS or controls (lower panels). Anti-CD3-induced proliferation, and IL-4 and IL-5 production in chronic progressive MS or controls, were not affected by anti-IL-12 (data not shown).

The IL-12-dependent increase in anti-CD3-induced IFN-γ in chronic progressive MS we observed could be secondary either to increased IL-12R expression on activated T cells in MS or increased biologically active IL-12 production by non-T cells in MS patients. It has been reported that recombinant IL-12 (rIL-12) can provide a costimulatory effect for activated human T cells that express detectable levels of IL-12R, particularly after mitogen stimulation (8, 10). To investigate these possibilities, we first studied IL-12R expression on anti-CD3 activated T cells. However, we found no difference between the number of IL-12R positive activated T cells in eight chronic progressive MS patients (22.6 ± 3.3%) vs. seven control subjects (21.4 ± 4.0%).

We thus investigated whether the raised IFN-γ secretion was secondary to increased IL-12 production in MS patients. To investigate this possibility, we added anti-CD3 to PBMC and measured secretion of IL-12, IL-4, and IL-2 in culture supernatants. As shown in Table 1, there was a marked increase in IL-12 production in chronic progressive MS subjects, which was not observed either in control subjects or relapsing–remitting MS subjects. No changes were observed in IL-4 or IL-2 secretion. To determine whether the amount of increased IL-12 observed in MS patients (24 pg/ml) was sufficient to affect IFN-γ production, we added rIL-12 to cultures of PBMC from control subjects in conjunction with anti-CD3 stimulation. We found that 10 pg/ml of rIL-12 increased anti-CD3-

Table 1. Increased T cell receptor-mediated IL-12 production in progressive MS

	IL-12, pg/ml	IL-4, pg/ml	IL-2, pg/ml
Control	1.5 ± 1.0 (17)	39.1 ± 7.7 (17)	719 ± 196 (7)
RR MS	3.7 ± 14 (9)	37.9 ± 10.4 (9)	ND
CP MS	24.8 ± 7.7 (15)*	44.5 ± 9.1 (15)	905 ± 279 (10)

PBMC were stimulated with anti-CD3 mAb as described, and supernatants were collected 24 h later. The levels of IL-12p70, IL-4, and IL-2 were measured by ELISA and expressed in pg/ml. Data are presented as mean ± SEM (number of subjects tested) for controls, relapsing–remitting MS patients (RR MS) and chronic progressive MS patients (CP MS).

*IL-12 secretion in CP MS patients vs control subjects ($P = 0.003$) and vs. RR MS patients ($P < 0.05$).

induced INF-γ production 3-fold and this was reversed by neutralizing anti-IL-12 Ab (data not shown).

Because anti-CD3 stimulates T cells and IL-12 is produced by non-T cells, we assumed that both cell populations were required for the increased IL-12 secretion in progressive MS patients. To identify whether one or both of the cell populations were abnormal in MS and which cells were responsible for the increased IL-12 in MS, we separated PBMC into T cell or non-T cell populations (APCs) and added them separately to plates coated with immobilized anti-CD3. IL-12 secretion in progressive MS patients was only observed when all three components were present: T-cells, APCs, and anti-CD3 (data not shown). Given this, we then mixed separated populations of T-cells and APCs from MS and normal subjects and measured IL-12 secretion. As shown in Fig. 2, minimal IL-12 was secreted by T cells plus APCs from control subjects whereas large amounts were secreted by MS subjects. However, when mixing experiments were performed, increased IL-12 was only observed when T cells from MS patients were cultured with APCs either from MS patients or controls. No increased IL-12 was observed when APCs from MS patients were cultured with T cells from controls subjects. These results clearly demonstrate that T cells from MS patients are responsible for the increased secretion of IL-12 in progressive MS, which act by inducing the non-T cell population to produce IL-12. To determine which T cell populations were involved, we separated T cells into CD4- and CD8-depleted populations; as shown in Fig. 2, we found that depletion of CD4 cells abrogated increased IL-12 secretion whereas depletion of CD8 cells had no effect.

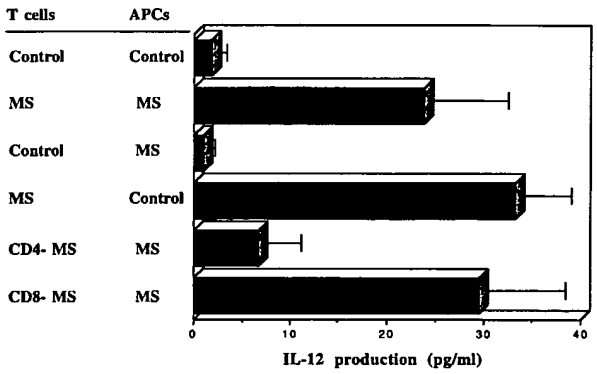

FIG. 2. Activated T cells but not APCs from progressive MS patients are responsible for increased IL-12 production. PBMC from control subjects and progressive MS patients were separated into APCs (non-T cells), T cells, CD4-depleted T cells ($CD4^-$), or CD8-depleted T cells ($CD8^-$). T cells or their subsets (1×10^6) from the control subject or from a chronic progressive MS patient were then activated with immobilized anti-CD3 mAb in the presence APCs (5×10^5) of either control subject or MS patient. The levels of biologically active IL-12p70 was measured in 24-h culture supernatants by ELISA and are expressed in pg/ml. Data represented by four top bars are mean ± SEM of six independent experiments with different control subjects and MS patients. Data represented by two bottom bars are mean ± SEM of four independent experiments with four different MS patients. APCs from MS patients produced significantly more IL-12 when cultured with activated T cells from MS patients vs. T cells from control subjects ($P < 0.025$). APCs from control subjects also secreted more IL-12 when cultured with activated T cells from MS patients vs. controls ($P < 0.001$). APCs from MS patients produced more IL-12 when cultured with autologous CD8-depleted ($CD8^-$) T cells vs. CD4-depleted ($CD4^-$) T cells ($P < 0.05$). No detectable IL-12 (less then 2 pg/ml) was secreted by APCs alone or T cells alone activated with immobilized anti-CD3, or T cells plus APCs in the presence of immobilized isotype control mouse IgG2a.

Although we found that the interaction between anti-CD3 activated $CD4^+$ T cells from MS patients with APCs was responsible for raised IL-12 production in CP MS, the nature of the T cell/APC interaction was unknown. To investigate this, we attempted to block IL-12 secretion in chronic progressive MS using mAb directed against cell surface structures known to be involved in T cell interaction with APCs. As shown in Fig. 3*a*, anti-CD40 ligand mAb completely blocked increased production of IL-12 in patients with progressive MS. No effect of anti-CD40 ligand Ab was seen on IL-4 production in the same culture supernatants. In addition, we found no significant effect on IL-12 production in MS using other Ab or soluble adhesion molecules to block interaction of LFA-1, CD2, or CD28/CTLA-4 (cytolytic T lymphocyte-associated antigen) expressed by T cells with appropriate ligands expressed by APCs (data not shown). These results suggested that anti-CD3 mAb was inducing increased CD40 ligand expression on T cells from MS patients, but not controls. As shown in Fig. 3*b*, this indeed was the case as a small, but significant increase in CD40 ligand expression occurred in anti-CD3 activated T cells from progressive MS patients but not in controls or relapsing–remitting patients. The expression of CD40 ligand occurred preferentially on activated $CD4^+$ T cells, although a small number of CD40 ligand positive $CD8^+$ T cells was also detected. Because optimal CD40 ligand expression may occur at earlier time points, we investigated the kinetics of CD40 ligand expression in purified T cells from MS patients and controls. As shown in Table 2, CD40 ligand expression first became evident at 24 h and was only observed in progressive MS patients. Although CD40 ligand is required for IL-12 production, other T cell-secreted cytokines may act synergistically with CD40 ligand. We have found that neutralizing anti-IFN-γ antibody reduced, though did not abolish, IL-12 secretion. Additional experiments were performed using phorbol 12-myristate 13-acetate (PMA) plus ionomycin, which is one of the strongest inducers of T cell activation. PMA plus ionomycin dramatically up-regulated CD40 ligand expression on T cells to a similar extent in both control subjects and MS patients with peak expression (56–78%) at 4 h after stimulation.

There are two series of investigations describing anti-CD3-induced CD40 ligand expression by human peripheral blood T cells. In the first, T cells were isolated by positive separation with sheep red blood cells (11–13). This separation led to CD2/LFA3 (lymphocyte function-associated antigen 3) interaction and CD2 engagement on the surface of T cells during separation. It has been reported recently that CD2/LFA3 interaction augments the expression of CD40 ligand on activated human $CD4^+$ T cells (14). Presumably this explains why the level of CD40 ligand expression by T cells was higher in these studies than in our studies. In contrast, when T cells from normal individuals were activated without previous CD2 engagement (15), stimulation of peripheral blood T cells through CD3 by mAb immobilized on plates induced only a minimal expression of the CD40 ligand, that was analogous to our system. Of note is that the phenotype of T cells directly after isolation from normal individuals vs. CP MS did not show generalized activation as measured by increased IL-2R,

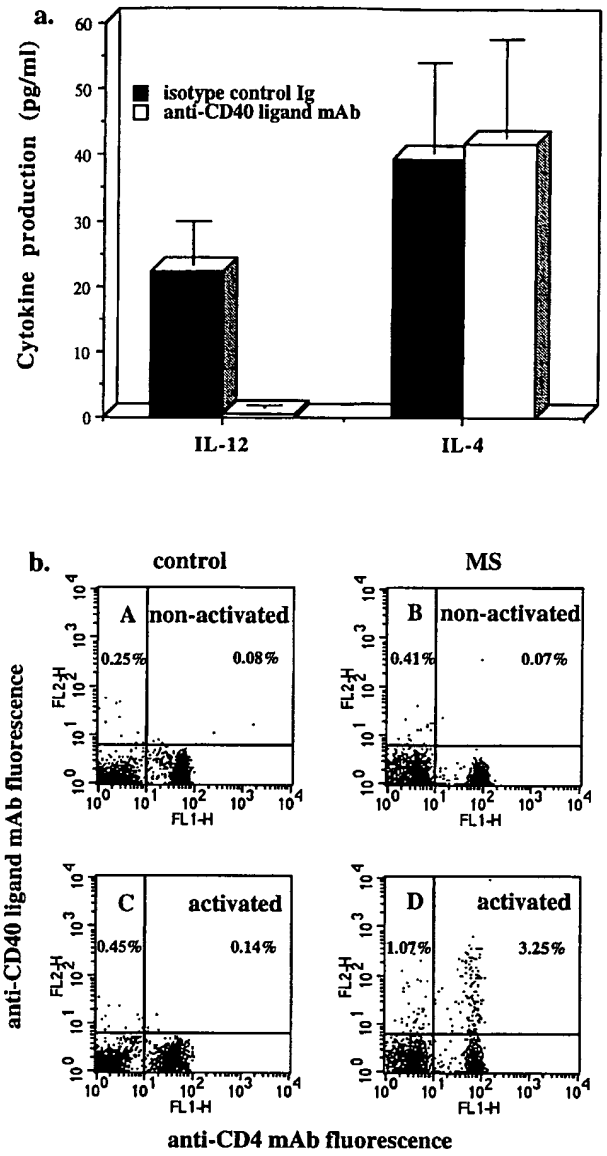

FIG. 3. (*a*) Increased TcR-mediated IL-12 production in MS is mediated by activated T cells via CD40 ligand. T cells and APCs from chronic progressive MS patients were activated with immobilized anti-CD3 mAb as described in the legend for Fig. 2. Anti-CD40 ligand mAb (10 μg/ml, clone m92, mouse IgG2a, provided by W. Fanslow, Immunex) or isotype control mouse IgG2a (10 μg/ml; PharMingen) were added from the beginning of the cultures. The levels of biologically active IL-12p70, IL-4, and IFN-γ were measured in 24-h culture supernatants by ELISA in pg/ml. Data are mean ± SEM of seven different experiments with different MS patients. Production of IL-12 was significantly reduced in the presence of anti-CD40 ligand mAb ($P = 0.007$ vs. isotype control Ig). Production of IFN-γ was 1381 ± 302 pg/ml in cultures with control mouse IgG2a and 634 ± 188 pg/ml in cultures with anti-CD40 ligand mAb. (*b*) Increased CD40 ligand expression by activated T cells in progressive MS. T cells from a control subject (*A* and *C*) or chronic progressive MS subject (*B* and *D*) were cultured with immobilized mouse IgG2a (*A* and *B*) or immobilized anti-CD3 (*C* and *D*) for 20 h. T cells were then stained with FITC-conjugated mAb specific for CD4 (Coulter Immunology) and phycoerythrin-conjugated anti-CD40 ligand mAb (PharMingen), according to the manufacturer's recommendations. Flow cytometric analysis of 5×10^3 cells from each sample was performed on an FACSort flow cytometer (Beckton Dickinson) according to standard procedures. Data are representative of six separate experiments. For all tested subjects, a significantly higher number of anti-CD3 activated T cells from progressive MS patients expressed CD40 ligand (2.5 ± 0.3%, $n = 6$) vs. controls (0.6 ± 0.2%, $n = 8$) or relapsing remitting MS patients (0.6 ± 0.24%, $n = 6$), $P < 0.001$, chronic progressive MS vs. control or relapsing-remitting MS. Less than 0.2% of anti-CD3-activated T cells from any tested donor were positively stained with combination of control FITC- and phycoerythrin-conjugated mouse IgG (all from Coulter Immunology).

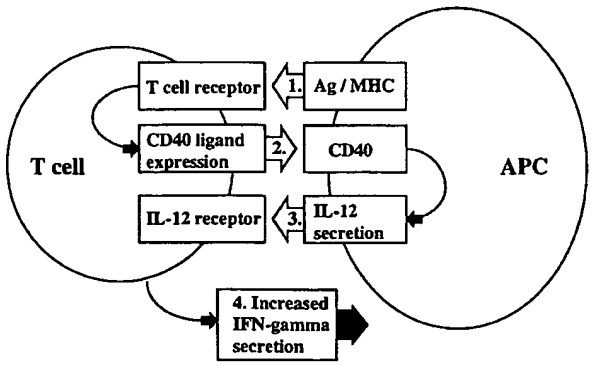

FIG. 4. Mechanism of increased IFN-γ secretion in progressive MS.

HLA-DR (human leukocyte antigen, DR region), or IL-12R expression (data not shown).

Our results provide an explanation for the altered regulation of IFN-γ in progressive MS patients and suggest that a self-perpetuating series of immune interactions occurs that results in a Th1-type immune response (see Fig. 4). The initiating immunologic event must involve repeated stimulation of the T cells through the Ag/major histocompatibility complex (MHC)/TcR complex, because stimulation through the TcR is required for CD40 ligand expression. It is postulated that this occurs in the relapsing–remitting form of the illness when patients have repeated attacks before entering the progressive phase. Although the trigger for these attacks is unknown, epidemiologic studies suggest that attacks are related in part to repeated viral infections and an increased number of attacks early in the disease is a poor prognostic sign (9, 16, 17). Increased expression of CD40 ligand on T cells then triggers the APC through CD40, an interaction that is known to induce IL-12 secretion by APCs (18–20). IL-12 then acts on T cells to induce the secretion of IFN-γ. Once secreted, IFN-γ itself acts on APCs to further up-regulate IL-12 secretion and perpetuate the cycle leading to a chronic state of Th1-type immune activation. It remains to be determined the degree to which increased CD40 ligand expression in MS is related to an inherent defect in MS T cells or is secondary to the state of T cell differentiation following chronic *in vivo* activation.

The association of IL-12 and CD40 ligand expression with raised IFN-γ secretion in MS is consistent with what is known about the effect of IL-12 and CD40 ligand on Th1-type autoimmune diseases in animals (21, 22). Thus, administration of IL-12 induces the rapid onset of insulin-dependent diabetes mellitus (23) in the NOD mouse. In the experimental autoimmune encephalomyelitis (EAE) model, animals treated with IL-12 *in vivo* have a more severe and prolonged form of EAE whereas anti-IL-12 reduces the incidence and severity of adoptively transferred EAE (24). Administration of IL-12 enhances, in an IFN-γ-dependent fashion, collagen-induced arthritis (25). Biologically active IL-12 secretion has not been studied in human autoimmune diseases in which it would be expected that enhanced production would be associated not only with disease progression but with increased IFN-γ production. This indeed was found in our MS patient population. IL-12 has also been studied in other human disease states including HIV, where IL-12 production was decreased and

Table 2. Kinetics of CD40 ligand expression

Time after activation, h	Percentage of T cells expressing CD40 ligand		
	Control	RR MS	CP MS
0	0.05	0.10	0.26
6	0.08	0.17	0.64
12	0.10	0.11	0.80
24	0.14	0.17	2.58
36	0.07	0.14	3.14
48	0.94	0.43	6.45

Purified T cells were activated with immobilized anti-CD3 as described in the legend for Fig. 3*b*. The percentage of T cells that expressed CD40 ligand was determined at various time points after activation. Results shown are representative of three independent experiments. RR MS, relapsing–remitting MS; CP MS, chronic progressive MS.

exogenous rIL-12 was reported to restore HIV specific cell-mediated immunity *in vitro* (26, 27).

CD40 ligand is a 33-kDa type II glycoprotein which is transiently expressed on the surface of T cells following activation (28). In the murine system, CD40 ligand is involved in T cell-dependent induction of nitric oxide, TNF-α (29), and IL-12 (18) secretion by macrophages and activated differentiated Th1 cells express a 20-fold greater amount of CD40 ligand than activated nondifferentiated T cells (30). In murine collagen-induced arthritis, disease is blocked by Ab to CD40 ligand (22). In addition, it has recently been shown by Gerritse *et al.* (31) that CD40–CD40 ligand interactions play an important role in EAE as treatment of animals with anti-CD40 ligand mAb completely prevented development of the disease. Furthermore, these investigators reported an increased number of $CD4^+$ T cells expressing CD40 ligand in MS patient brain sections (31). Our results demonstrate that functional immune abnormalities in the blood of MS patients are linked to CD40–CD40 ligand interactions and are related to disease progression. Thus, increased IL-12 production in progressive MS is mediated by activated T cells via CD40 ligand, a mechanism that has not been previously described in human disease states. Of note is that Stuber *et al.* (32) have recently observed CD40 ligand-mediated increased IL-12 and Th1-type responses in the mouse model of 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Also, Grewal *et al.* (33) have recently reported impairment of antigen-specific T cell priming in mice lacking CD40 ligand expression and postulated that antagonists of CD40–CD40 ligand interaction may be of benefit in the treatment of autoimmune diseases such as multiple sclerosis.

In summary our findings establish the presence of immune activation associated with raised IFN-γ in MS which is linked to IL-12 production by non-T cells and to CD40 ligand expression by $CD4^+$ T cells. These immune abnormalities are seen most prominently in patients with the progressive form of the disease, suggesting an important link to disease pathogenesis and progression. Furthermore, these findings not only provide an immunological basis to understand disease mechanisms in MS but identify two molecules that may serve as targets for treatment of the disease.

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1. Martin, R., McFarland, H. F. & McFarlin, D. E. (1992) *Annu. Rev. Immunol.* **10,** 153–187.
2. Beck, J., Rondot, P., Catinot, L., Falcoff, E., Kirchner, H. & Wietzerbin, J. (1988) *Acta Neurol. Scand.* **78,** 318–323.
3. Lu, C.-Z., Jensen, M. A. & Arnason, B. G. W. (1993) *J. Neuroimmunol.* **46,** 123–128.
4. Panitch, H. S., Hirsch, R. L., Haley, A. S. & Johnson, K. P. (1987) *Lancet* **i,** 893–895.
5. Woodroofe, M. N. & Cuzner, M. L. (1993) *Cytokine* **5,** 583–588.
6. Trinchieri, G. (1995) *Annu. Rev. Immunol.* **13,** 251–276.
7. Windhagen, A., Newcombe, J., Dangond, F., Strand, C., Woodroofe, M. N., Cuzner, M. L. & Hafler, D. A. (1995) *J. Exp. Med.* **182,** 1985–1996.
8. Desai, B. B., Quinn, P. M., Wolitzky, A. G., Mongini, P. K. A., Chizzonite, R. & Gately, M. K. (1992) *J. Immunol.* **148,** 3125–3132.
9. Matthews, W. B., Acheson, E. D., Batchelor, J. R. & Weller, R. O. (1985) in *McAlpine's Multiple Sclerosis*, ed. Matthews, W. B. (Churchill Livingstone, Edinburg), pp. 49–72.
10. Chizzonite, R., Truitt, T., Desai, B. B., Nunes, P., Podlaski, F. J., Stern, A. S. & Gately, M. K. (1992) *J. Immunol.* **148,** 3117–3124.
11. Patel, H. R., Oshiba, A., Jeppson, J. D. & Gelfand, E. W. (1996) *J. Immunol.* **156,** 1781–1787.
12. Splawski, J. B., Nishioka, J., Nishioka, Y. & Lipsky, P. E. (1996) *J. Immunol.* **156,** 119–127.
13. Spriggs, M. K., Armitage, R. J., Strockbine, L., Clifford, K. N., Macduff, B. M., Sato, T. A., Maliszewski, C. R. & Fanslow, W. C. (1992) *J. Exp. Med.* **176,** 1543–1550.
14. Karmann, K., Hughes, C. C. W., Fanslow, W. C. & Pober, J. C. (1996) *Eur. J. Immunol.* **26,** 610–617.
15. Lane, P., Traunecker, A., Hubele, S., Inui, S., Lanzavecchia, A. & Gray, D. (1992) *Eur. J. Immunol.* **22,** 2573–2578.
16. Panitch, H. S. (1994) *Ann. Neurol.* **36,** S25–S28.
17. Sibley, W. A., Bamford, C. R. & Clark, K. (1985) *Lancet* **i,** 1313–1315.
18. Kennedy, M. K., Picha, K. S., Fanslow, W. C., Grabstein, K. H., Alderson, M. R., Clifford, K. N., Chin, W. A. & Mohler, K. M. (1996) *Eur. J. Immunol.* **26,** 370–378.
19. Shu, U., Kinawa, M., Wu, C. Y., Maliszewski, C., Vezzio, N., Hakimi, J., Gately, M. & Delespesse, G. (1995) *Eur. J. Immunol.* **25,** 1125–1128.
20. Kato, T., Hakamada, R., Yamane, H. & Nariuchi, H. (1996) *J. Immunol.* **156,** 3932–3938.
21. Trembleau, S., German, T., Gately, M. K. & Adorini, L. (1995) *Immunol. Today* **16,** 383–386.
22. Durie, F. H., Fava, R. A., Foy, T. M., Aruffo, A., Ledbetter, J. A. & Noelle, R. J. (1993) *Science* **261,** 1328–1330.
23. Trembleau, S., Penna, G., Bosi, E., Mortara, A., Gately, M. K. & Adorini, L. (1995) *J. Exp. Med.* **181,** 817–821.
24. Leonard, J. P., Waldburger, K. E. & Goldman, S. G. (1995) *J. Exp. Med.* **181,** 381–386.
25. Germann, T., Szeliga, J., Hess, H., Storkel, S., Podlaski, F. J., Gately, M. K., Schmitt, E. & Rude, E. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4823–4827.
26. Clerici, M., Lucey, D. R., Berzofsky, J. A., Pinto, L. A., Wynn, T. A., Blatt, S. P., Dolan, M. J., Hendrix, C. W., Wolf, S. F. & Shearer, G. M. (1993) *Science* **262,** 1721–1724.
27. Chehimi, J., Starr, S. E., Frank, I., D'Andrea, A., Ma, X., MacGregor, R. R., Sennelier, J. & Trinchieri, G. (1994) *J. Exp. Med.* **179,** 1361–1366.
28. Fanslow, W. C., Srinivasan, S., Paxton, R., Gibson, M. G., Spriggs, M. K. & Armitage, R. J. (1994) *Semin. Immunol.* **6,** 267–278.
29. Stout, R. D., Suttles, J., Xu, J., Grewal, I. S. & Flavell, R. A. (1996) *J. Immunol.* **156,** 8–11.
30. Roy, M., Waldschmidt, T., Aruffo, A., Ledbetter, J. A. & Noelle, R. J. (1993) *J. Immunol.* **151,** 2497–2510.
31. Gerritse, K., Laman, J. D., Noelle, R. J., Aruffo, A., Ledbetter, J. A., Boersma, W. J. A. & Claassen, E. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2499–2504.
32. Stuber, E., Strober, W. & Neurath, M. (1996) *J. Exp. Med.* **184,** 693–698.
33. Grewal, I. S., Xu, J. & Flavell, R. A. (1995) *Nature (London)* **378,** 617–620.